Ph.D. DISSERTATION

HIGH-THROUGHPUT RETRIEVAL OF MOLECULAR CLONES FOR POLYNUCLEOTIDE SYNTHESIS

폴리뉴클레오타이드 합성을 위한
초고속 분자 클론 추출 기술 개발

BY

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지도교수 권 성 훈

이 논문을 공학박사 학위논문으로 제출함

2012 년 12 월

서울대학교 대학원

전기컴퓨터 공학부

김 효 기

김효기의 공학박사 학위논문을 인준함

2012 년 12 월

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Abstract

Molecular clone is a large population of DNA replicated from a single DNA molecule. Production, analysis and retrieval of the molecular clones are the key techniques in current biotechnology. Here, high-throughput method for the manipulation of molecular clones is presented using highly parallel sequencing and radiation-based non-contact molecular clone retrieval system.

Specifically, production and analysis methods of the molecular clones are investigated using a highly parallel sequencing platform. In emulsion polymerase chain reaction (PCR) at the sample preparation of parallel pyrosequencing, large amount of molecular clones are created. After the production of large amount of molecular clones, individual molecular clone is sequenced so that sequence and position information of each molecular clone are identified. Molecular clone retrieval system based on non-contact energy transmission is developed for rapid and precise retrieval of each molecular clone. Pulse laser, optical component, vision module, and motorized stage are integrated in the system. Also, system control

software is developed combining functions such as: (i) extraction of sequence and positional information of each molecular clone from output data of highly parallel sequencing, (ii) scanning sequencing plate and high-resolution image reconstruction, (iii) algorithmic identification of molecular clone in the reconstructed image, (iv) synchronization of sequencing plate movement, pulse-laser radiation, and carrier plate movement.

Furthermore, retrieval method of molecular clones produced by bacterial cell is presented. Mixture of genetically diverse bacterial cell stock is spread and grown on a solid growth medium. After the short period of growth, position of each cell clone is identified using microscope vision scanning system, and then each cell clone is retrieved using focused pulse laser. Retrieved individual cell clone is contained in carrier micro well plate. DNA of retrieved individual cell clone is tagged with barcode DNA, and analyzed and identified by highly parallel sequencing.

Method and system developed here may be applicable in a wide range of biotechnology area such as synthetic DNA production, antibody drug discovery, and functional genome study with exceptional cost reduction and increased throughput due to highly parallel nature of the method.

Keywords: Molecular cloning, Gene synthesis, Synthetic biology, Systems biology.

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Chapter 1

Introduction

Molecular clone is a large population of DNA replicated from a single DNA molecule. Manipulation of molecular clones (i.e. production, analysis and retrieval of the molecular clones) has been used for a wide range of current biotechnology. For example, a starting step for recombinant protein expression is the construction of recombinant DNA and following molecular cloning in bacterial cells. Also, producing and sequence verification of molecular clones are crucial steps in synthetic gene construction. Since chemically synthesized DNA has inherent errors such as deletion, addition, or transversion of bases, causing low yield to find error-less long DNA, molecular cloning and subsequent sequence verification is essential process when producing long DNA.

Conventional method for the manipulation of molecular clone is based on the

following techniques: (i) preparation of vector DNA and template DNA to be cloned, (ii) recombinant plasmid synthesis using restriction endonuclease and ligase, (ii) insertion of recombinant plasmid into bacterial cells, (iii) spread and growth of the bacterial cells on solid growth medium, (iv) selection of bacterial colony with pick and place fashion, (v) re-growth of the colony, plasmid extraction and sequence verification of the plasmid using chain-termination sequencing (so called 'Sanger sequencing'). Conventional molecular cloning developed in the early 1970s dramatically changed the understanding of basic science and made a breakthrough in biotech industry. [1-6] However, while functional genome study or ultra-long DNA construction in today's biotechnology requires massive amount of production and analysis of molecular clones, low throughput of the pipette-based colony pick-and-place and subsequent sequence verification using chain-termination sequencing limits the progress.

Recently, dramatic advancement on sequencing technology enables the analysis of large amount of DNA with 100 mega-bases to 100 giga-bases sequencing throughput. [7-16] In comparison to conventional chain-termination sequencing which processes 96 to 192 samples per run, today's parallel sequencing based on sequencing-by-synthesis or sequencing-by-ligation (so called 'next generation sequencing (NGS)') analyze 1 million to 1 billion different fragments of DNA in a single sequencing run. Besides the sequencing throughput, per base sequencing cost

is significantly dropped. For example, while sequencing cost of the conventional chain-termination sequencing is $2,000 per mega-bases, sequencing cost of the next generation sequencing is $10 to $0.1 per mega-bases. Generally, the throughput of current NGS is 3 to 6 orders of magnitude higher and the cost of NGS is 3 to 4 orders of magnitude lower than conventional Sanger sequencing. If this massively parallel sequencing can be applied to the manipulation of molecular clones, it would dramatically increase the throughput and decrease the cost and labor.

Furthermore, conventional method for the construction of template DNA desired to be cloned is based on polymerase chain reaction (PCR) with a primer set targeting specific region of biological DNA such as genomic DNA or RNA in certain cells or assembly of short fragment of chemically synthesized DNA. In either PCR primer or short fragment DNA for the long DNA assembly, starting material is chemically synthesized DNA. Conventional chemical DNA synthesis is based on phosphoramidite solid-phase synthesis method. Most of the commercial DNA synthesis machine utilizes columns filled with resin where the synthesis reaction is performed. The synthesis machine produces 96 to 384 different DNAs per synthesis run. Recent genome scale study requires large amount of different DNAs, but the low throughput and high cost nature of the conventional method is the bottleneck. Recently, many researches show that the microarray DNA can be used for the construction of long DNA molecules. DNA microarray has been typically used for

the genome scale gene expression study. Methods to fabricate DNA microarray is highly parallel, producing 10,000 to 1,000,000 of different DNA on a single substrate per a synthesis run. Significant cost drop and superior throughput of microarray DNA over conventional column-based synthetic DNA makes microarray DNA a good option for the large scale construction of template DNAs. [17-24]

After the generation of molecular clones, desired molecular clones are individually separated and contained for downstream experiment such as sequencing or screening. Conventional retrieval method of molecular clones utilizes pick-and-place method. In conventional method, bacterial cells are first spread on solid growth medium. After overnight growth of the colony, researcher identifies each molecular clone with naked-eye and gently picks small colony with pipette. Small amount of colony is resuspended in liquid growth medium. These experimental steps are called ‘colony picking’. In a commercial automated colony picker, tips similar with a manual pipette tip are attached on a robotic arm. Macro-scale vision system identifies each molecular clones and colony picking event is performed with robot control. Conventional contact-based colony picking is labor intensive and slow due to the tip exchange or tip washing process. Also, throughput is limited due to the macro-scale vision system which typically identifies and picks 100 colonies in a solid growth plate. If colony retrieval would utilize non-contact method with high-resolution vision system, throughput of the retrieval may increase due to the reduced

growth time, dense colonies in a single solid growth plate, and without the need of tip exchange and washing steps.

This thesis presents a high-throughput technique for the manipulation of molecular clones. With the integration of the recent advancement of highly parallel sequencing and synthesis of DNA, thesis introduces a method to create highly diverse molecular clones, to analyze individual molecular clones, and to retrieve the molecular clones.

This work is organized as follows: First, I review the basic concepts of the fundamental techniques and discuss the limitation of the conventional techniques in chapter 2. Process of conventional molecular cloning, column-based chemical DNA synthesis, DNA microarray fabrication, chain-termination sequencing, and highly parallel sequencing are introduced. In chapter 3, a system overview and essential technical component for the high-throughput manipulation of molecular clones are presented. Detail pipeline of the process including (i) production of highly diverse molecular clones using in-vitro and in-vivo methods, (ii) sequence verification of molecular clones using highly parallel sequencing, and (iii) pulse-laser based molecular clone retrieval system, will be introduced. In chapter 4, sample preparation method for the molecular clone will be presented. Designing microarray DNA, emulsion PCR for the amplification bias reduction, and molecular clone identification using next generation sequencing will be introduced. In chapter 5,

molecular clone retrieval system based on non-contact energy transmission is presented for rapid and precise retrieval of each molecular clone. Pulse laser, optical component, vision module, and motorized stage are integrated in the system. Also, system control software is developed combining functions such as: (i) extraction of sequence and positional information of each molecular clone from output data of highly parallel sequencing, (ii) scanning sequencing plate and high-resolution image reconstruction, (iii) algorithmic identification of molecular clone in the reconstructed image, (iv) synchronization of sequencing plate movement, pulse-laser radiation, and carrier plate movement. In chapter 6, microfluidic DNA concentrator is designed for the downstream experiment.

Chapter 2

Fundamentals

The understanding of research motivation and a system developed in thesis requires the understanding of previous methods and basic principles of currently available techniques. The basic concepts of these methods needed for the following chapters will be briefly introduces here, starting with conventional molecular cloning. Current synthetic DNA production methods of column-based DNA synthesis and microarray DNA synthesis methods will be introduced in the second part of this chapter. In later part of the chapter, currently available DNA sequencing methods will be introduced including chain-termination sequencing and highly parallel sequencing method such as sequencing-by-ligation and sequencing-by-synthesis.

2.1 Conventional Molecular Cloning

2.1.1 Definition of Molecular Clone

Molecular clone is a large population of DNA replicated from a single DNA molecule. A single copy of DNA can be amplified either with *in-vivo* amplification by vector cloning or *in-vitro* amplification by polymerase chain reaction (PCR). Typically, molecular cloning refers to an *in-vivo* amplification including recombinant vector construction and transformation into cell.

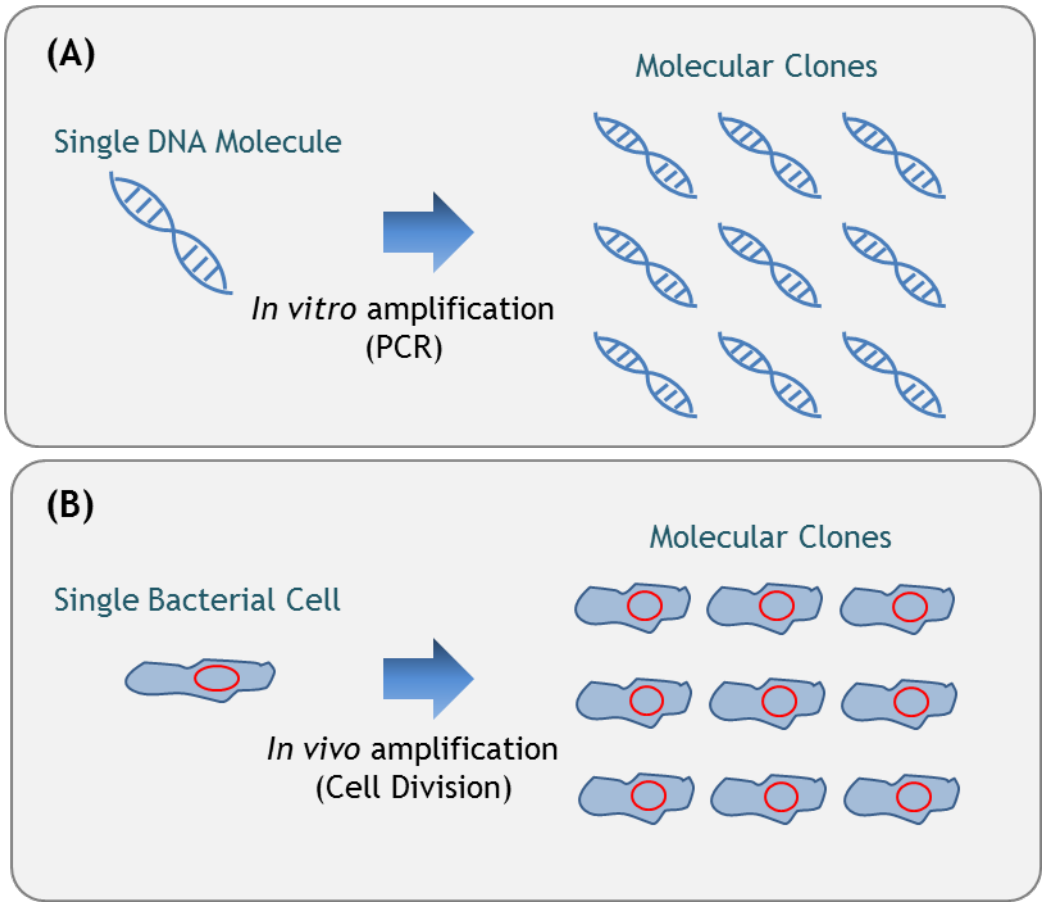


Figure 2.1 Schematic diagram of molecular clone. Molecular clone is a large population of DNA replicated from a single DNA molecule.

2.1.2 Polymerase Chain Reaction

In 1980s, Dr. K. Mullis et al. developed a method of in-vitro DNA amplification (PCR: Polymerase Chain Reaction). [5], [6] More specifically, PCR is referred as 'primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase'. PCR requires a series of temperature changes, called cycles. There are 2 or 3 discrete temperature steps in each temperature change cycle.

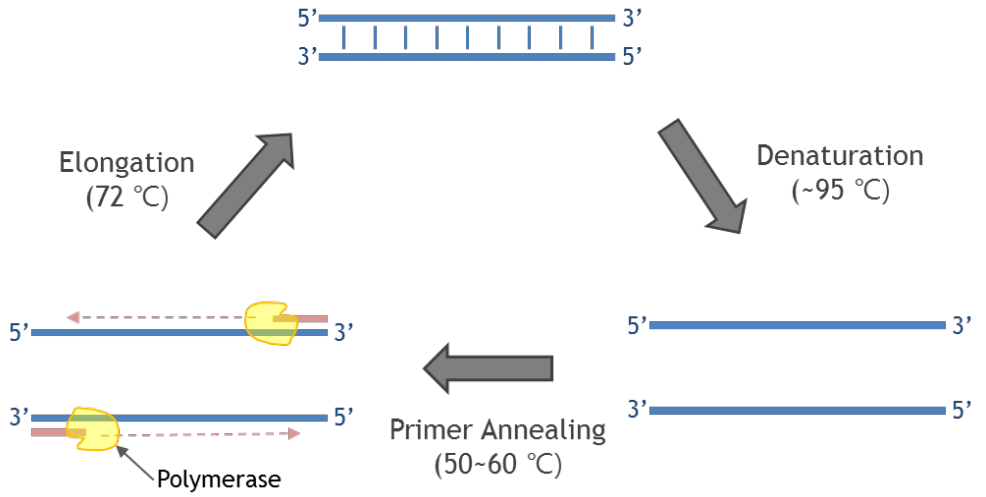


Figure 2.2 Polymerase Chain Reaction: Cyclic thermocycling and exponential amplification of template DNA.

The cycling is often started by a denaturation step at a high temperature (>90°C). In denaturation step, double stranded DNA is melted and linking of hydrogen bonds between two strands is dissociated. After the denaturation, temperature is cooled down for the primer annealing. Determination of an annealing temperature requires the consideration of melting temperature (Tm) of the primers. Successful PCR for a specific band amplification starts from successful primer hybridization. Typical

temperature range of primer annealing is around 50 ~ 60 ℃. Polymerase enzyme binds and waits for elongation at the 3' end overhang of primer-template complex. After the annealing of primers, temperature is raised to 68 ~ 72 ℃ so that the polymerase can synthesize a new strand. Deoxynucleoside triphosphates (dNTPs) are the building-blocks from which the DNA polymerase synthesizes a new DNA strand. As the number of cycle of denaturation-annealing-elongation increases, copy number of the template DNA is exponentially amplified. With perfect amplification yield, copy number of the template DNA is given by following: $\mathrm{T} = \mathrm{N} \times 2^n$, where T stands for total number of amplified templates, N for initial template copy number, n for the number of PCR cycle. Maximum copy number of template to be amplified is limited by the copy number of primers. Also, too much PCR cycle yields error-containing products meaning that polymerase is prone to incorporate undesired dNTPs to the template sequence.

Besides PCR, rolling circle amplification (RCA) is another method for *in-vitro* amplification. RCA is suitable in template with tandem repeat. Also, RCA typically is performed at isothermal state so thermocycling is not required.

2.1.3 Vector Cloning

Vector cloning is a method for the *in-vivo* amplification. Cloning vector is a small piece of DNA that can carry foreign DNA into a host cell. Typically foreign DNA should have a same overhang with the carrier vector so that those two sticky ends

can be joined with ligase enzyme.

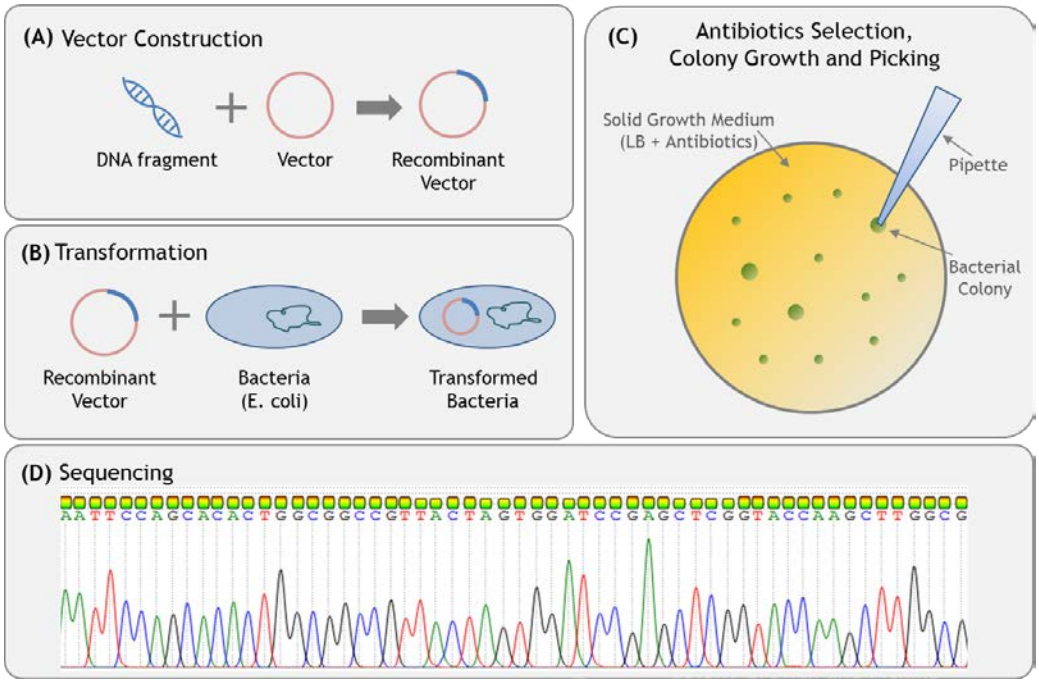


Figure 2.3 Conventional vector cloning method. A single DNA fragment is incorporated in a vector DNA which forms recombinant vector. The recombinant vector is inserted in a bacterial cell and the cell is grown in growth medium with appropriate antibiotics.

In vector cloning process, vector construction and subsequent insertion of the recombinant vector into bacterial cell is required. One method to insert synthetic vector into a host cell is to give a heat shock with chemically competent cell and recombinant vector mixture under 42℃. After plasmid insertion, bacterial cells are spread in a solid growth medium so that the distance between the cells should be sparse enough not to overlap after the colony formation. Typical growth time to form colony takes overnight. Bacterial colony is a visible cluster of bacteria growing

on the surface of the solid-growth medium.

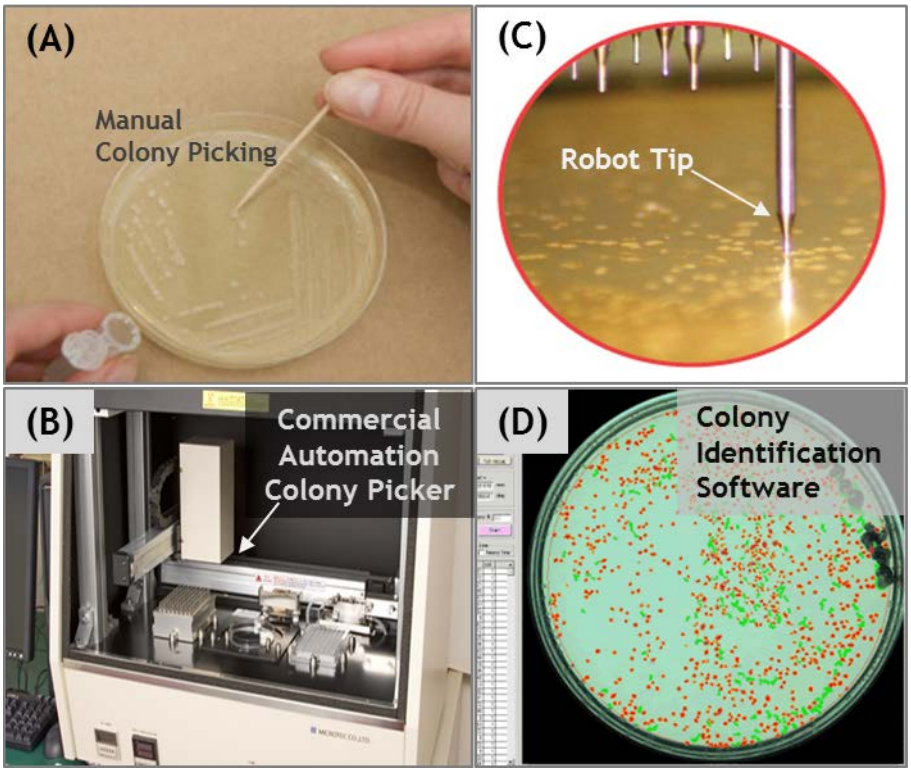


Figure 2.4 Conventional colony picking method. (A) manual colony picking with pipette. (B) Automated commercial colony picker head. (C) Automated colony picker machine. (D) Software of automated colony picker

All cells within the colony are divided from a single ancestor cell. Mutation can occur in the cell division process, but the mutation rate is so low in *in-vivo* replication system that it is considered that the DNAs carrying inside the cell are identical.

2.1.4 Retrieval of Molecular Clones

After the growth of the colony, researcher identifies each molecular clone with

naked-eye and gently picks small colony with pipette. Small amount of colony is resuspended in liquid growth medium. These experimental steps are called ‘colony picking’. In a commercial automated colony picker, tips similar with a manual pipette tip are attached on a robotic arm. Macro-scale vision system identifies each molecular clones and colony picking event is performed with robot control. [4]

2.1.5 Discussion of the Conventional Molecular Cloning

Conventional contact-based colony picking is labor intensive and slow process due to the tip exchange or tip washing process. Also, throughput is limited by resolution of vision system and size of colony picking tip. Size of the picking tip is typically ~mm. If density of the clones in a growth plate is increased, clone-to-clone inter-clone distance decreases resulting in cross-contamination. It is hard to separate more than 100 clones per plate in conventional colony picking experiment.

2.2 Column-based Chemical DNA Synthesis

2.2.1 Structure of DNA

A nucleotide consists of a heterocyclic base such as adenine, cytosine, guanine or thymine. A pentose sugar ring, which in conjunction with a phosphate group, constitutes the sugar-phosphate-backbone of the DNA molecule. Subsequent nucleotides are linked via a phosphodiester bond between the 3’ and 5’ carbons of the deoxyribose sugars. The sugar-phosphate-backbone also determines the

orientation of a DNA strand. The two strands of double helix are oriented in opposite directions.

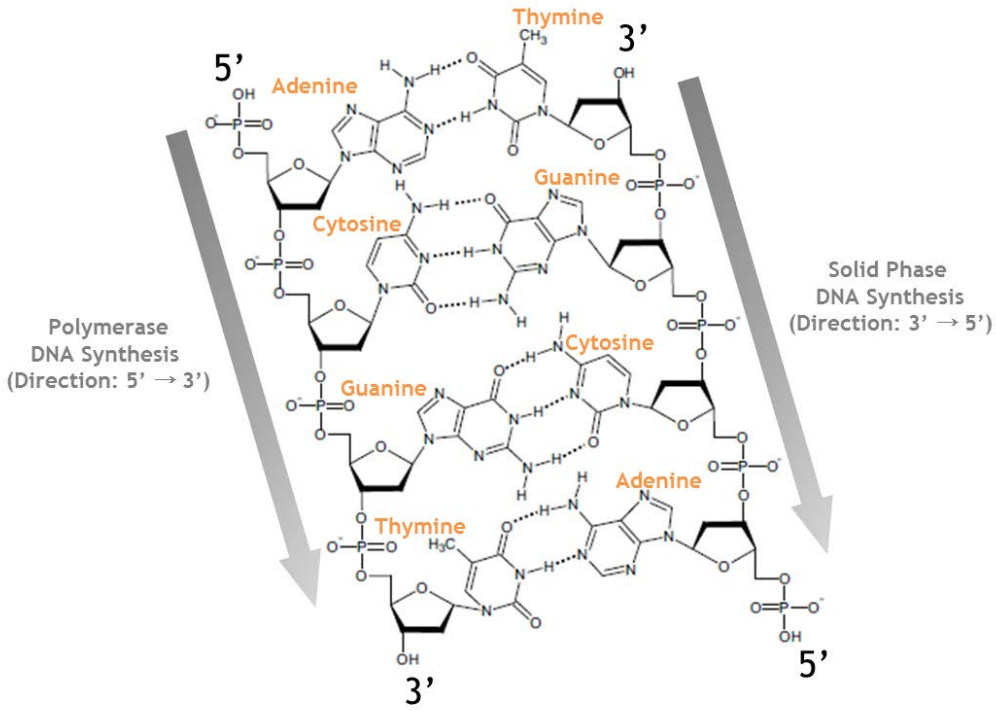


Figure 2.5 Chemical structure of double stranded DNA. The stability of double strand DNA originates from hydrogen bond between complementary base pairs adenine-thymine and guanine-cytosine

2.2.2 Phosphoramidite DNA Synthesis Chemistry

In 1980s, Marvin H. Caruthers et al., developed a phosphoramidite synthesis method for chemically synthesizing DNA, which became a standard protocol for current synthetic DNA chemistry. [25-27] Phosphoramidite DNA synthesis proceeds in the 3′

to 5′ direction. One nucleotide is added per synthesis cycle. In each cycle, the solutions are pumped through the reaction column. At the beginning of oligonucleotide synthesis, the first base-protected nucleoside is attached to the resin (typically controlled pore glass). The resin-bound nucleoside has a 5′ 4,4'-dimethoxytrityl (DMT) protecting group. The DMT group prevents polymerization during cycle of synthesis proceeds, and this DMT protecting group must be removed from the resin-bound nucleoside before oligonucleotide synthesis can proceed. This removal of DMT group is called detritylation. After the detritylation, the resin-bound nucleoside is ready to react with the next nucleotide. The coupling of a new base cannot produce a perfect 100% yield due to the nature of chemical synthesis. Highest coupling yield is around 99.5% and the coupling yield decreases as the cycle of the synthesis accumulates. There are a few unreacted 5' hydroxyl groups on the resin-bound nucleotide. In capping step, reagents such as acetic anhydride and N-methylimidazole make 5' hydroxyl group inert to subsequent base addition reaction. Without the capping step, 5' hydroxyl group would be reactive to the subsequence base incorporation, thus producing base lack at the final product. Deletion of the base would produce a complex mixture of DNA and most of the DNA would contain deletion error. After the capping step, unstable phosphite-triester bonds should be converted to a stable phosphate trimester bonds. This conversion is called 'oxidation'. This 'deprotection-coupling-capping-oxidation'

cycle is repeated so as to synthesize desired length of DNA. After the synthesis, the linker attaches to the 3′-end of the oligonucleotide to the solid support should be cleaved. The linker used in typical DNA synthesis is the succinyl linker and the linker is cleaved by concentrated ammonium hydroxide treatment. After the cleavage, the synthesized oligonucleotide dissolved in ammonium hydroxide is heated to remove the protecting groups of the bases and phosphates.

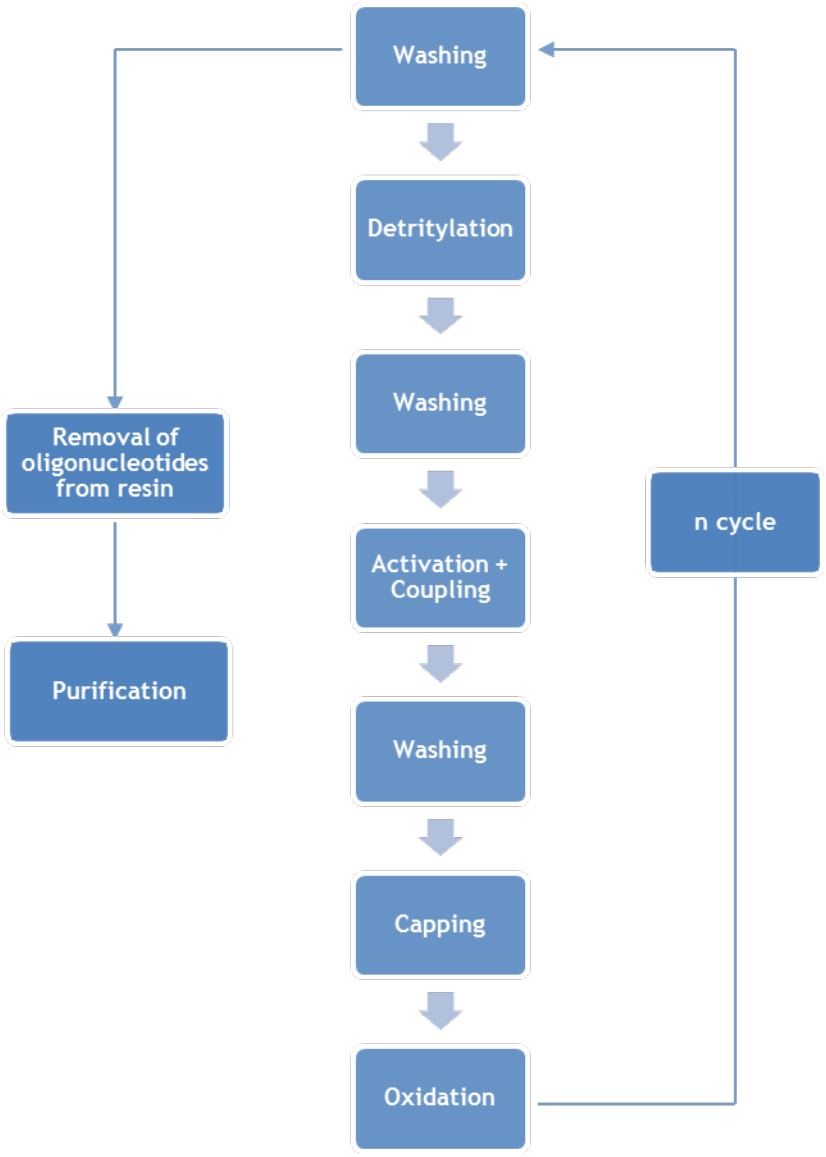


Figure 2.6 Chemical oligonucleotide synthesis process

2.2.3 Commercial Column-based DNA Synthesizer

Automated commercial DNA synthesizer is available. For example, multi-column DNA synthesizer: *Mermade 192*(www.bioautomation.com), it synthesizes 192

oligonucleotides per a synthesis run. Similar like inkjet, reagents are dispensed from the top of the column. [30] In the column there is CPG for DNA immobilization and growth. Coupling efficiency is about 99.5%. Running time is about 5 to 6 hours for 35bp oligonucleotide synthesis. Cost of the machine is around $180,000. Running cost for the synthesis of 50nmole scale volume is $0.05/base for 20mer. Commercial column-based DNA synthesizer produces large amount of DNA from nmole to μmole. However, the synthesis of large number of different sequences with the column-based DNA synthesizer requires a lot of time and money for the synthesis and purification.

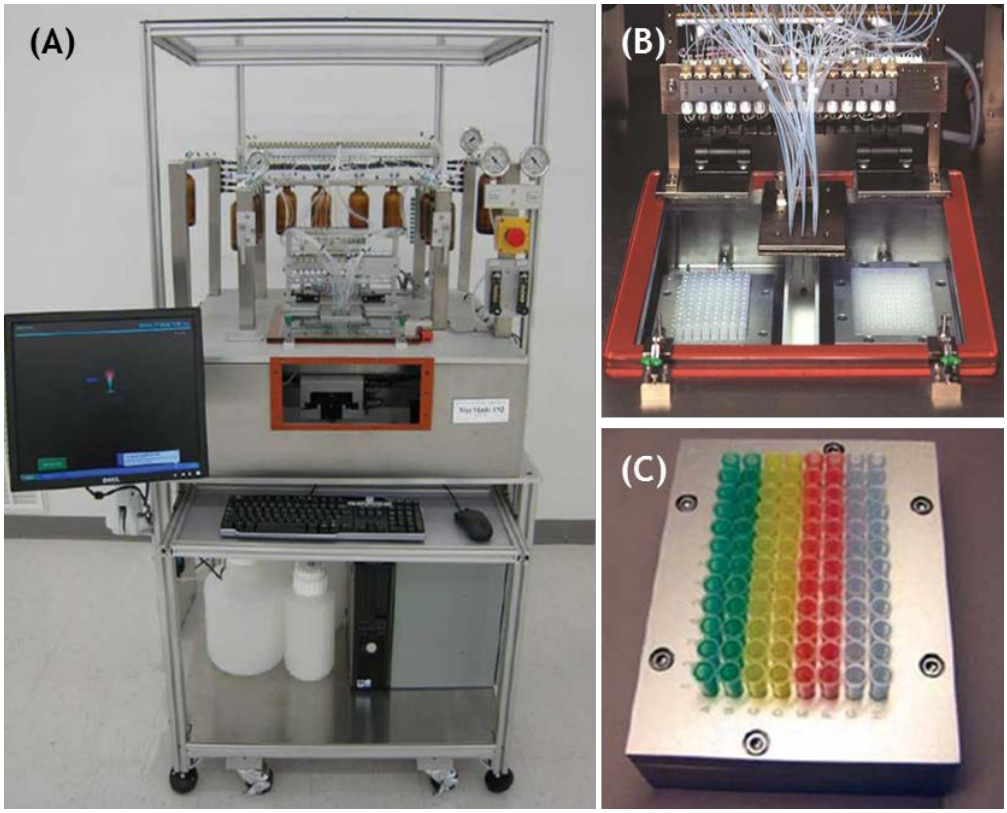


Figure 2.7 Commercial column based DNA synthesizer. 192 oligonucleotides are synthesized per a synthesis run. (A) Commercial machine, (B) Nozzle head, (C) Column chuck.

2.3 DNA Microarray

2.3.1 Fabrication Method for DNA Microarray

A DNA microarray is a collection of microscopic DNA spots attached to a solid surface. DNA microarray contains 10,000 to 1,000,000 different oligonucleotides in a solid carrier (typically glass slide). [27-29] The synthesis of the DNA microarray basically utilizes similar synthesis chemistry with previously described cyclic phosphoramidite-based method. The differences from column-based DNA

synthesizer are the deprotection mechanism (light-based synthesis) and small volume localized reagent delivery (inkjet-based or electrochemical synthesis). In light-based microarray DNA synthesis initially developed around 1990 by Fodor et al., short oligonucleotides length of 25bp are synthesized on the surface of the microarray. Spatially addressable photo-deprotection chemistry (MeNPOC or NPPOC phosphoramidite) enables highly parallel synthesis of *de novo* DNA sequences. In inkjet-based synthesis, synthesizer uses piezoelectric small drop deposition, and the system is used for *in-situ* synthesis of arbitrary oligonucleotide. Also, electrochemical *in-situ* synthesis method is reported, where spatial addressing of deprotection is controlled by CMOS addressable array with localized acid generation.

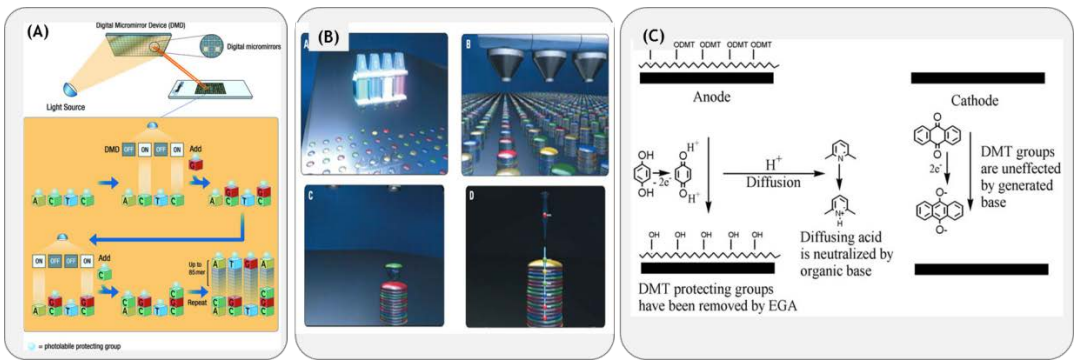


Figure 2.8 Microarray DNA synthesis methods. (A) light-based synthesis, (B) Inkjet-based synthesis, (C) Electrode array based synthesis [Figure from company web information site: *Nimblegen*, *Agilent*, *Combimatrix*]

Traditionally, DNA microarray is used for the gene expression monitoring and

gnome scale SNP analysis. Since 10,000 ~ 1,000,000 different oligonucleotides are synthesized in parallel, synthesis cost per oligonucleotide drops significantly. Also, this parallel synthesis process is fast so that typically 12 hours of synthesis run produces a glass slide scale microarray DNA. In comparison, the cost of microarray oligonucleotides is significantly lower than that with the column-based synthesizer. In large scale gene synthesis, many researches have been done using microarray DNA-derived techniques.

2.3.2 Discussion on the Coupling Efficiency

As shown in the section 2.2.2 'Phosphoramidite DNA Synthesis Chemistry', oligonucleotide synthesized from solid phase synthesis is prone to contain errors. Major error source is a base deletion. In commercial DNA synthesis company report, Custom Inkjet printer (POSaM) claims 98% ~ 98.5% coupling efficiency. Mermaid 192 oligo synthesizer from *Bioautomation, Corp.* claims 99.4% coupling efficiency. Microarray DNA from *Agilent* claims 99.5% coupling efficiency using depurination process. The coupling yield is crucial for building long oligo length over 100bp.

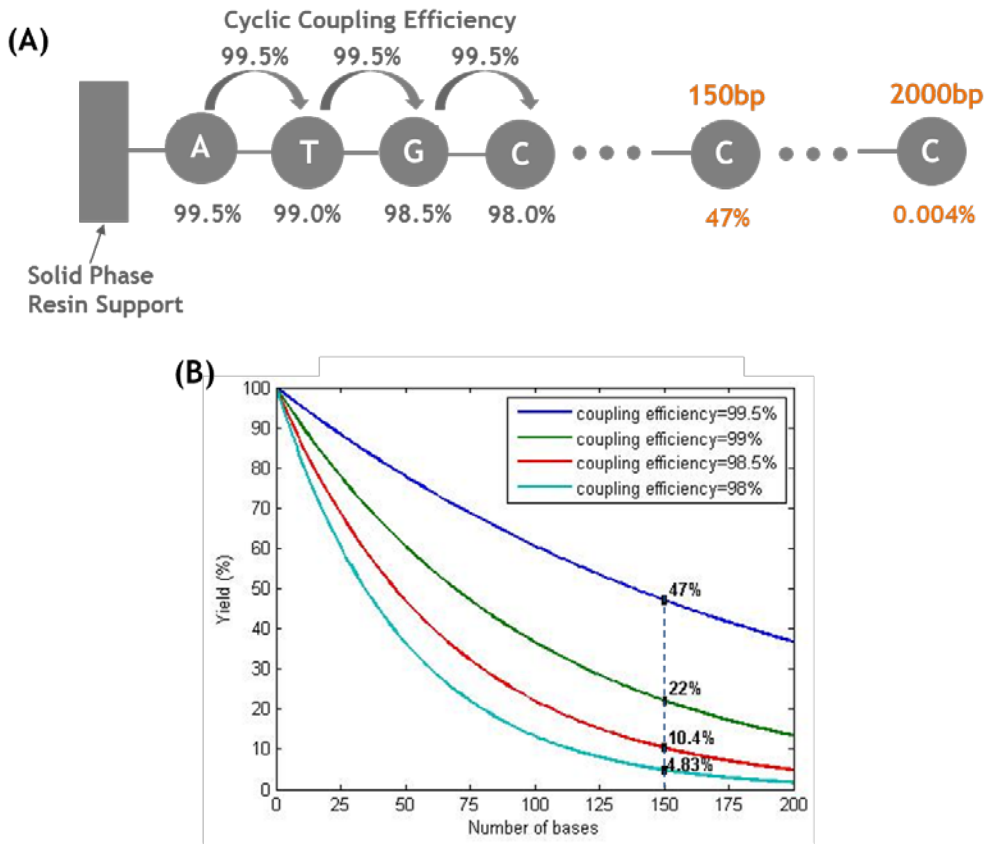


Figure 2.9 Yield profile when synthesizing chemical DNA. (A) Schematic of cyclic solid phase synthesis, (B) yield profile.

Total yield to produce correct DNA can be expressed as follows: $Y = y_{cpl}^{N_{base}}$, where Y stands for total yield of error-less DNA, Y_{cpl} for the cyclic coupling efficiency and N_{base} for the number of bases to be synthesized. As total yield is expressed in exponential form, the yield to produce long perfect DNA is dramatically reduced. For example, total yield drops to 36.7% when synthesizing 200bp oligonucleotide. If one would synthesize oligonucleotide with length of

2,000bp, total yield drops to 0.0044% meaning that most of the oligonucleotides produced in the synthesis reaction are error-containing.

2.4 Chain-termination Sequencing

Chain-termination sequencing is a method of DNA sequencing, based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase. [7] Chain-termination sequencing is also called ‘Sanger sequencing’ which is developed by F. Sanger et al. in the late 1970s. The classical chain-termination method requires a single-stranded DNA template, a DNA primer, DNA polymerase, normal deoxynucleotide triphosphates (dNTPs), and dideoxynucleotide triphosphates (ddNTP) that terminate DNA strand elongation. ddNTP lacks a 3'-OH group which is required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to stop synthesis of DNA when a ddNTP is incorporated. The ddNTP can be fluorescently labeled for detection in automated sequencing machines using capillaries, argon-ion laser, and CCD. The cost of chain-termination sequencing is around $5 per sample.

2.5 Highly Parallel Sequencing

Recently, dramatic advancement on sequencing technology enables the analysis of large amount of DNA with 100 mega-bases to 100 giga-bases sequencing throughput. In comparison to conventional chain-termination sequencing which processes 96 to

192 samples per run, today's parallel sequencing based on sequencing-by-synthesis or sequencing-by-ligation (so called 'next generation sequencing (NGS)') analyze 1 million to 1 billion different fragments of DNA in a single sequencing run. Besides the sequencing throughput, per base sequencing cost is significantly dropped. For example, while sequencing cost of the conventional chain-termination sequencing is $2,000 per mega-bases, sequencing cost of the next generation sequencing is $10 to $0.1 per mega-bases. Generally, the throughput of current NGS is 3 to 6 orders of magnitude higher and the cost of NGS is 3 to 4 orders of magnitude lower than conventional Sanger sequencing.

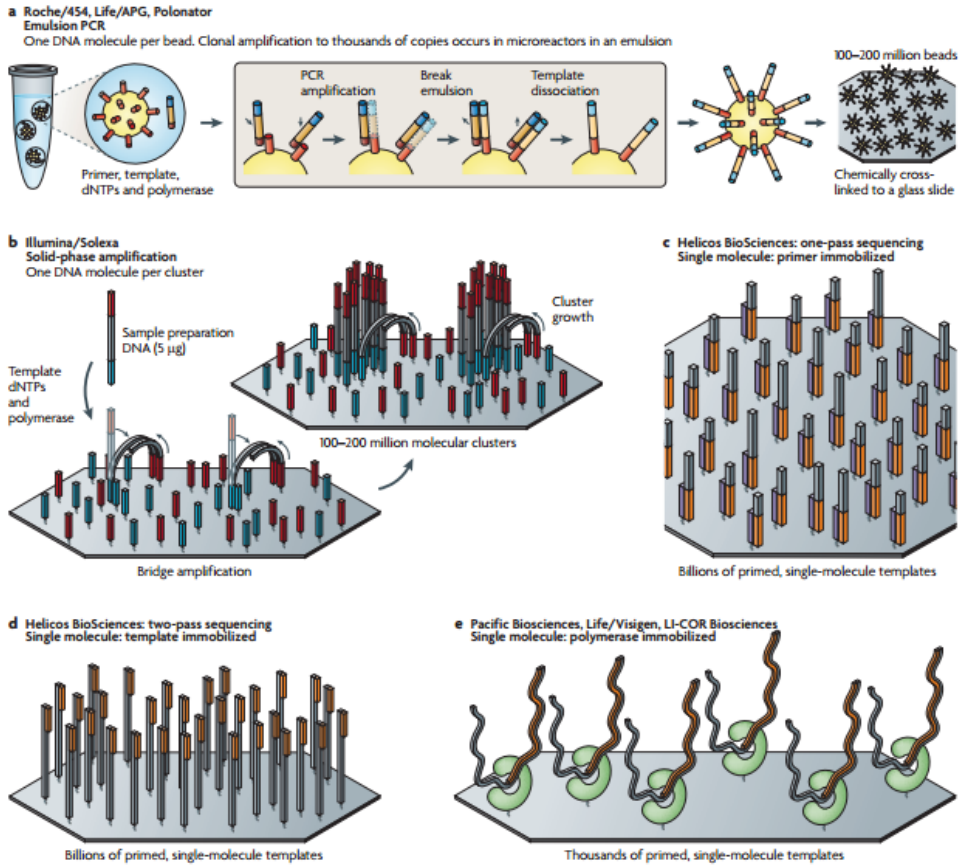


Figure 2.10 Next generation sequencing techniques [19]

2.5.1 Sequencing-by-Ligation

Sequencing-by-ligation method is one of the first developed in high throughput sequencing area. Sequencing by ligation relies on the sensitivity of hybridization. Target DNA molecule with a single stranded unknown sequence is joined with one end by a known sequence (called ‘adapter’ sequence). A mixed pool of fluorescently labeled short probe DNA typically length of 8 ~ 9 bases is then incorporated in the target DNA. DNA ligase joins the adapter sequence and the perfectly hybridized

probe DNA. After the ligation of adapter DNA and probe DNA, flow cell is washed and automated stage with microscope scans the entire flow cell and calls the unknown sequence. The probe has cleavable fluorescent molecule so that the next probe incorporation is performed after the cleavage of the linker between probe and the fluorescent molecule. Typically template DNA is attached on a bead and millions of beads were subject to the cycles of sequencing run (sequencing cycle: probe incorporation-wash-scan-cleavage-wash-next probe incorporation).

2.5.2 Sequencing-by-Synthesis

Sequencing-by-synthesis method is the most widely used approaches these days. A common sequencing-by-synthesis method is to use DNA polymerase to extend DNA strands in parallel. Sample preparation of sequencing-by-synthesis starts with the creation of large population of molecular clones using emulsion PCR or bridge PCR amplification. One target DNA molecule is amplified and either attached on a bead or a sequencing plate. After the creation of molecular clones, each clone is then sequenced. Nucleotides are provided either one at a time so that the unknown sequence is identified by the incorporation of dNTPs as extension proceeds. A method of *454 Life Science* is based on a pico liter plate carrying millions of molecular clones attached on beads. A method of *Illumina* is based on a reversible terminator linker and bridge amplified molecular clones.

2.6 Comparison of Sequencing Platforms

Performance metrics of DNA sequencing platforms are categorized as follows: sequencer cost (instrument cost, $), sequencing yield per run (Mb or Gb/run), read number per run (million reads or billion reads/run), read length (500bp), sequencing cost per run ($/run), run time (hr). For example, ‘read number per run’ can be a number of molecular clones in each run. *454 GS FLX* platform produces 1 million reads per run, and there are 1 million molecular clones (attached on bead) are present in pico liter plate.

Platform	454 FLX+	454 GS Junior	Illumina GAIIx	Illumina HiSeq2000	Illumina MiSeq
Instrument Cost	$500,000	$108,000	$256,000	$654,000	$128,000
Sequence yield per run	400-600Mb	35Mb	30~90Gb	600Gb	1.5~2Gb
Read number per run	1 million	0.1 million	300 million	3 billion	4 million
Read length	700bp	500bp	150bp	150bp	150bp
Sequencing cost per run	$6,200	$1,100	$17,575	$23,470	$1,160
Cost/Mb	$7	$22	$0.19	$0.04	$1
Run Time	24hr	10hr	10days	30-10days	26hr

Platform	Ion Torrent Proton	Ion Torrent PGM	PacBio RS	3730xl (Sanger Sequencing)	
Instrument Cost	$149,000	$75,000	$695,000	$99,000	
Sequence yield per run	10~100G	40Mb~1Gb	100Mb	1.9~84kb	
Read number per run	50~200 million	1.6~4 million	0.01 million	96	
Read length	200bp	200bp	1~10kb	400~900bp	
Sequencing cost per run	$1,000	$750	$300-1,700	$144	
Cost/Mb	$0.09	$7 (314 chip), $2 (316 chip)	$7-38	$2,308	
Run Time	2-4hr	2~4hr	2hr	3hr	

Figure 2.11 Comparison of NGS performance

Chapter 3

System Overview

Chemical gene synthesis is the basic technique for the design of enhanced biochemical production pathway and functional genome study. Various genes are necessary to investigate protein functions and combination of different genes is required to study protein-protein interaction for target identification. However, conventional gene synthesis produces high error rates from chemical synthesis of short oligonucleotides due to the cyclic nature of phosphoramidite reactions. A high error rate of oligonucleotide produced by cyclic chemical synthesis limits long DNA synthesis. Furthermore, it requires not only time-consuming and laborious cloning and Sanger sequencing but also expensive overall cost.

For example, total synthesis of artificial bacterial genome (size of 1 mega bases) requires around 10,000 different synthetic oligonucleotide length of 100bp. To

remove error of chemically synthesized oligonucleotide, number of cloning and Sanger sequencing verification is estimated to around 10,000 ~ 100,000. Also, studying functional genome relevant for cancer requires 1,000 modified genes with 1kb for each gene. Total length of modified gene for the study of cancer functional genomics is 1Mb. Order of work for today's challenging biotechnology requires massive amount of effort and resource. [20]

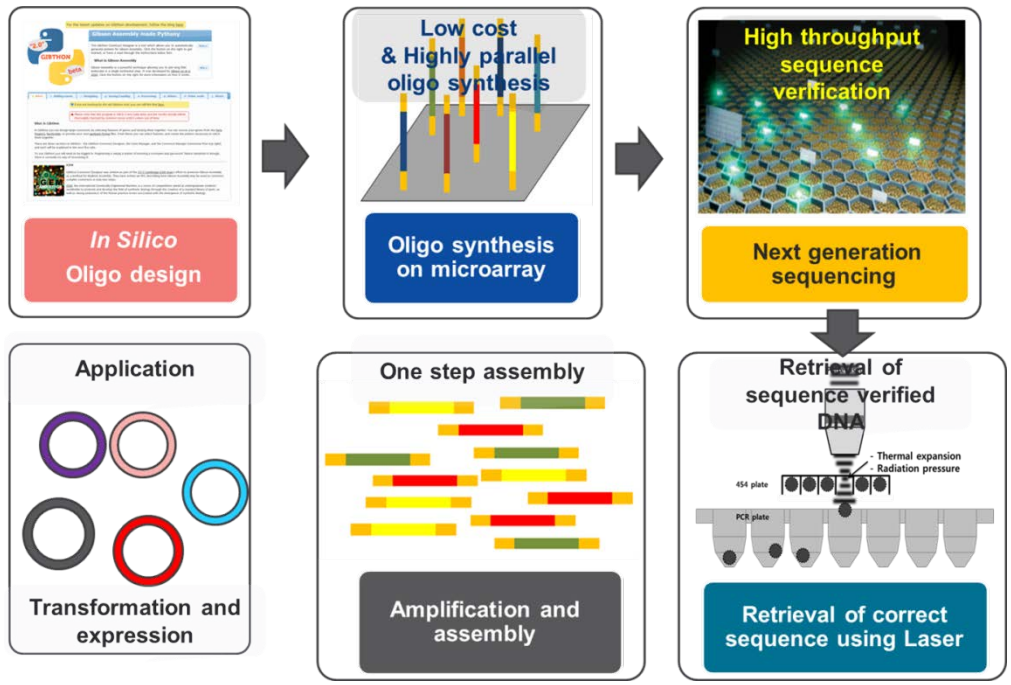


Figure 3.1 System overview of high throughput molecular clone manipulation.

In this work, as depicted in figure 3.1, I used microarray oligonucleotides pool containing millions of oligonucleotides to significantly lower down price of starting material. Based on the highly parallel molecular clone production and sequencing,

burdensome cloning and Sanger sequencing steps are not required. After sequencing, we mathematically map the beads containing error-free oligonucleotides with sequencing results and optically retrieve those beads with pulse laser retrieval system. Retrieved sequence verified oligonucleotides are PCR amplified for the downstream experiment.

Two very accurate, computer controlled motorized stages face each other holding NGS plate and PCR plate respectively. We randomly picked several cornerstone beads from local area. By Sanger sequencing of those cornerstone beads and comparing them with sequence information of NGS, we can link virtual location of sequence information to real world coordinate. Due to its imaging distortion of NGS machine, it is almost impossible to globally map real world location of wanted sequence beads. However, error from imaging distortion can be minimized in relatively small area where real world location of sequence verified microbead is predictable using linear transformation algorithm. Thermal expansion or radiation pressure of focused pulse laser plays a major role to retrieve microbeads that contains sequence verified oligonucleotides. In contrast to pick and place method, owing to non-contact nature of laser light, we can easily avoid cross-contamination and prompt retrieval process can be done within a seconds.

Chapter 4

Sample Preparation

4.1 Sequence Design for Microarray DNA synthesis

We use oligonucleotides from DNA microarray chip for the assembly of total sequence. Oligonucleotides should have the form including primer sites, restriction sites (EarI or BstI produces blunt end recognizing specific sequence on double strand DNA) and main body for assembly. Custom designed MATLAB code was generated for the large scale oligo design.

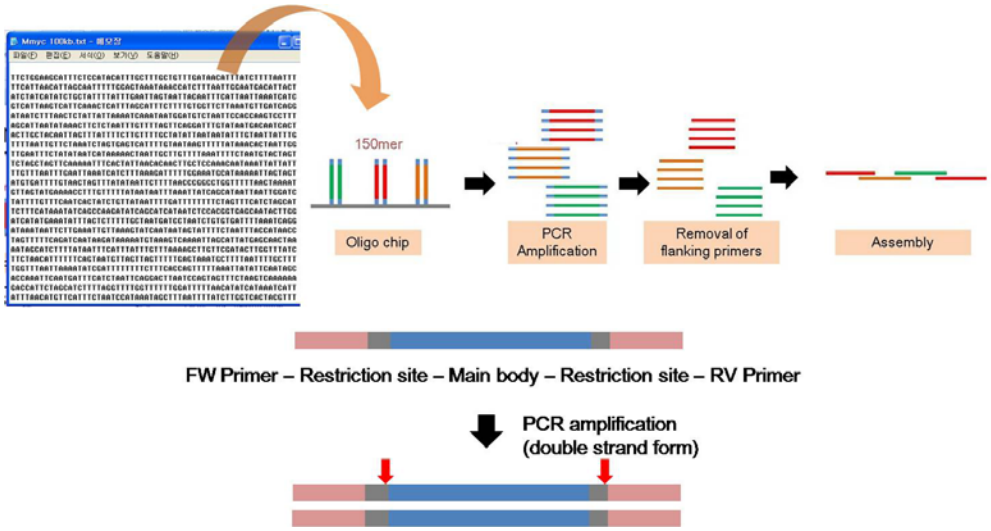


Figure 4.1 Construction of microarray DNA.

```
%% Oligo design for Long sequence synthesis %%
clear all

%% Sequence Concatenation
fid = fopen('Mouse_mitochondrial_genome_complete_sequence.dat');
rawSequence = textscan(fid, '%s','bufsize', 100000);

%% Oligos: Main assembly %%

fw_univ = 'AGGGCATCTTAGCGGTCG';           % forward universial primer
rv_univ = 'CCACACATGGCACCTTTG';           % reverse universial primer

fw_earI = 'CTCTTCT';                % forward earI restriction site
rv_earI = 'AGAAGAG';                % reverse earI restriction site

fw_flanking = strcat(fw_univ,fw_earI);
rv_flanking = strcat(rv_earI,rv_univ);
```

```
L_total = length(rawSequence{1,1}{1,1}); % Total length of gene
sequence
L_oligo = 100; % Length of microarray oligo
L_flanking = length(fw_flanking); % Length of flanking primer
sequences
L_overlap = (L_oligo-2*L_flanking)/2; % Length of overlap sequences
L_frag = L_oligo-2*L_flanking; % Length of main body sequences
N_frag = ceil(L_total/(L_frag-L_overlap)); % Total number of
fragments

oligo = fopen('SNU_full_overlap_Kutzneria_EarI.txt', 'w');

N_iter = N_frag;
 for n=1:N_iter-1
       if n < N_frag-1
           catSequence = strcat(rawSequence{1,1}{1,1}((n-1)*(L_frag-
L_overlap)+1:(n-1)*(L_frag-L_overlap)+L_frag));
           medCat = upper(strcat(fw_flanking,catSequence,
rv_flanking));      % Concatenation of overlap and main body

fprintf(oligo,'SNU_full_overlap_Kutzneria_EarI %u\t%s\n',n,medCat);
       else
           catSequence = strcat(rawSequence{1,1}{1,1}((n-1)*(L_frag-
L_overlap)+1:length(rawSequence{1,1}{1,1})));
           medCat = upper(strcat(fw_flanking,catSequence,
rv_flanking));      % Concatenation of overlap and main body

fprintf(oligo,'SNU_full_overlap_Kutzneria_EarI %u\t%s\n',n,medCat);
       end
```

```
    end


fclose(oligo);
```

4.2 Emulsion PCR for Reduced Amplification Bias

We analyzed 1852 oligonucleotides with 454 GS FLX sequencing platform. One of the key problems we found is the significant amplification bias. A certain oligonucleotides in 1852 pool have strong preference to be amplified than other oligonucleotides. Once easily-amplifiable-oligonucleotide dominates, they consume every dNTPs and primers. Thus, emulsion PCR is required for unbiased amplification. If biased oligonucleotide pool is analyzed and retrieved from NGS platform, coverage of desired oligonucleotides would be low.

4.3 Optimization of Emulsion PCR Condition

Emulsion size and template concentration may affect amplification bias when performing PCR of oligonucleotide pool. Quantitative measure of emulsion generation is required for setting optimal and reproducible protocol. Fluorescence labeled oligonucleotide is used for clear image processing.

Emulsion PCR was performed with different magnet rotation speed. (RPM 600, 900, 1200) Duration of rotation is fixed to 5min. After emulsion generation, samples were re-suspended on slide glass and many images were acquired in every experiment condition with fluorescence microscope. Size of emulsion was analyzed

in custom built MATLAB code.

```
%% Emulsion size characterization

clear all

W1 = imread('1200-1.tif'); % Input microscope picture
imshow(W1);
[centers1, radii1,metric1] = imfindcircles(W1,[1,60],
'Sensitivity', 0.5, 'EdgeThreshold',0.25);

W2 = imread('1200-2.tif'); % Input microscope picture
imshow(W2);
[centers2, radii2,metric2] = imfindcircles(W2,[1,60],
'Sensitivity', 0.5, 'EdgeThreshold',0.25);
viscircles(centers2, radii2,'EdgeColor','b');

W3 = imread('1200-3.tif'); % Input microscope picture
[centers3, radii3,metric3] = imfindcircles(W3,[1,60],
'Sensitivity', 0.5, 'EdgeThreshold',0.25);
```

In three rotation speed conditions, RPM 900 shows sharpest distribution peak and 1200RPM result shows there are smaller emulsion than other conditions. For successful emulsion PCR, template concentration and number of emulsion should be matched. After emulsion generation, emulsion was diluted 100x and dispensed on hematocytometer. Image processing was applied to count emulsions and analyzed. It is assumed that number of emulsion at our current emulsion generation protocol is

about 10^7. Also, template volume when generating emulsion is 200uL, average number of template in an emulsion is about 600 copy (divided by $6x10^9$).

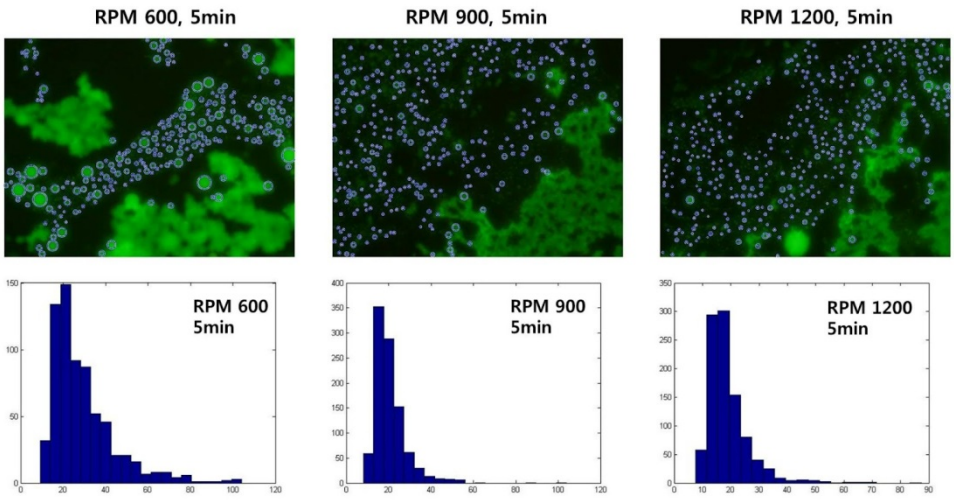
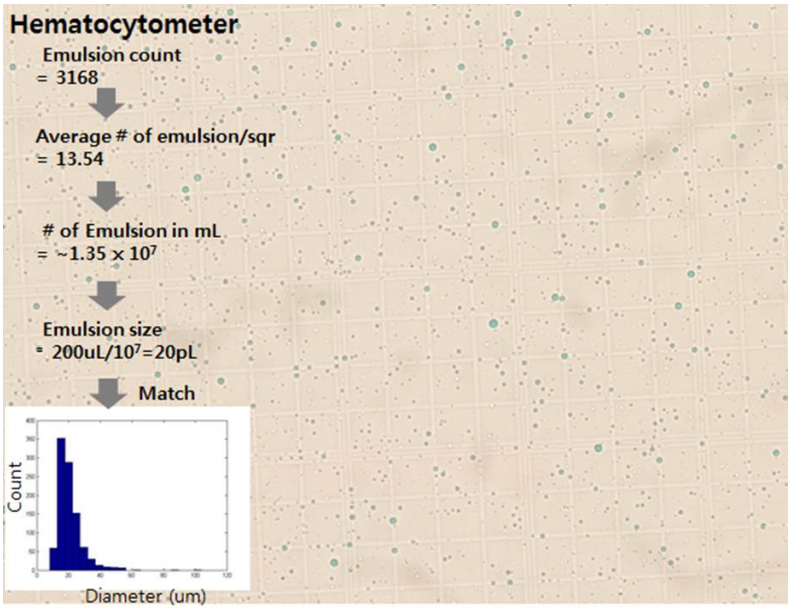


Figure 4.2 Optimization of emulsion PCR condition. Spin speed 900RPM shows the most narrow emulsion size distribution. Also, the mean size of each emulsion is around 10pL scale.



Template concentration	1ng/uL
Average dNTP Mw	487g
DNA length	200bp
Molar concentration	1.03E-08M
Copy number in 1uL	6.18E+09#/uL
AVR # of template in an emulsion	600#

Figure 4.3 Hematocytometer based emulsion condition optimization.

4.4 Adapter Ligation for Next Generation Sequencing

Conventional blunt ligation is performed using strategy 1 (phosphorylated DNAs). However, we found that there are significant adapter dimers and template-adapter by-products. Gel electrophoresis reveals that there are significant byproducts in phosphorylated adapter and phosphorylated template. Non-phosphorylated adapter inhibits adapter-adapter ligation. Big difference can be seen in ligated product

between strategy 1 and strategy 2. Still, there are byproducts which will reduce template coverage in NGS. Gel electrophoresis reveals that there are significant byproducts in phosphorylated adapter and phosphorylated template. There are significant difference in ligated product between strategy 1 and strategy 2. Undesired byproducts will reduce template coverage in NGS.

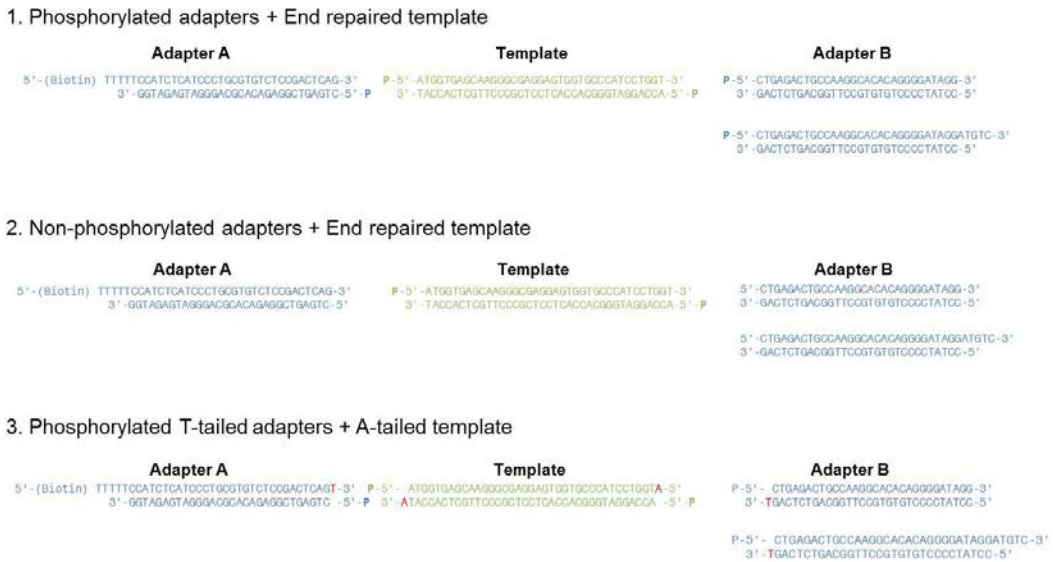


Figure 4.4 strategies for the adapter ligation: 1) phosphorylated adapters + phosphorylated template (blunt ligation), 2) non-phosphorylated adapters + phosphorylated template (blunt ligation), 3) phosphorylated T-tailed adapters + A-tailed template (TA ligation).

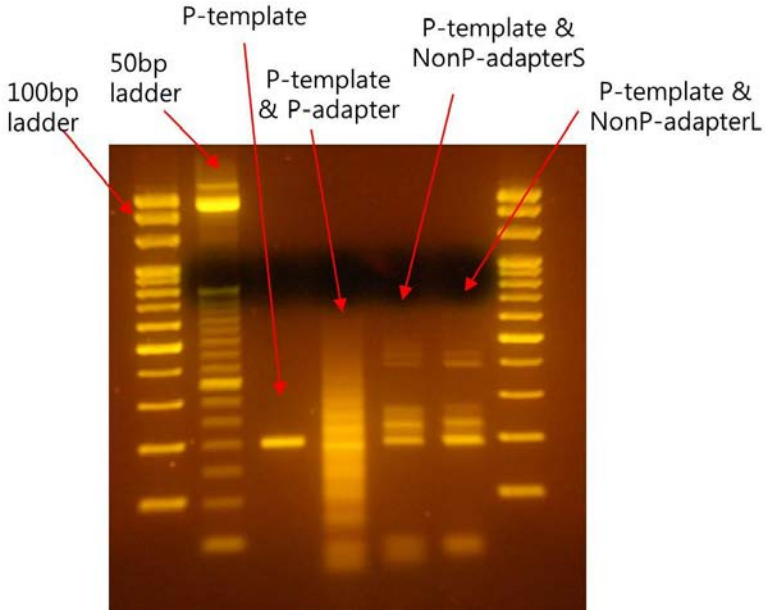


Figure 4.5 Gel electrophoresis study of adapter ligation.

Figure 4.6 Optimal adapter ligation strategy.

4.5 Comparison of the Emulsion PCR and Normal PCR

To test the sample preparation condition, 2 samples were analyzed with NGS. Sample 1 includes amplified microarray oligo pool. Both emulsion PCR product and normal PCR product were equally concentration-calibrated and pooled in sample 1. Sample 2 includes 4 different lengths of DNA. 720bp is GFP, 1.3kb is 5'UTR+GFP, 1.5kb is 5'UTR+GFP+SV40, and 2.5kb is MutS gene. These samples were weighted

concentration-calibrated and pooled. Gel electrophoresis QC before sequencing run indicates that we prepared samples correctly.

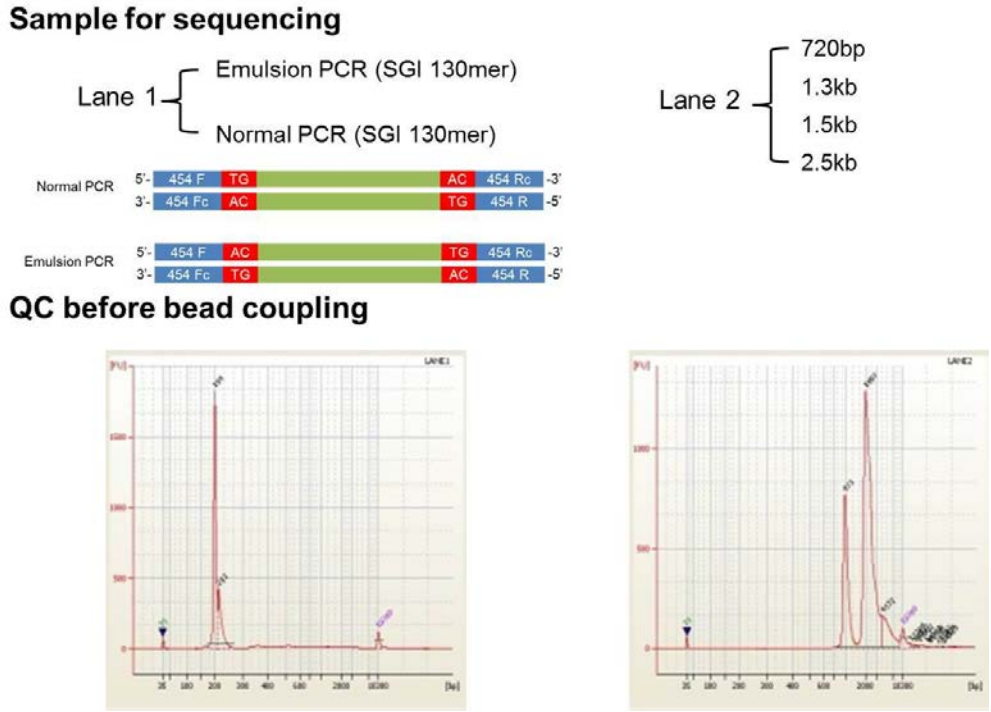


Figure 4.7 Sample distribution and gel electrophoresis QC. (Left-Down) Sample of 130bp template, (Right-Down) Sample of 720bp, 1.3kb, 1.5kb, 2.5kb mixture

Sequencing chemistry used in this run is FLX+ which is different from normal GS FLX. FLX+ (FLX plus) should produce longer read length of maximum 800bp, whereas normal FLX usually produces 400bp maximum. 454 GS FLX gives 1/8 lane produces 80~100k reads. 60k reads were produced in the experiment. Lane 1 shows clear read length distribution around 130bp. Lane 2 shows various read length distribution. Length of our sample is almost over the read length limit of 454 FLX+.

Analysis is performed with custom MATLAB code.

```
clear all

%% Read 454 files
 filename_2='454reads_2.dat';
fid_2 = fopen(filename_2);
A2 = textscan(fid_2,'%s');
 %% Read Oligo List

sgi130_2='gene_2500bp.txt';
fid_sgi130_2 = fopen(sgi130_2);
A_sgi130_2 = textscan(fid_sgi130_2,'%s');
A_sgi130_2 = upper(A_sgi130_2{1,1});

%% Sequence Find
 B2 = cell(length(A2{1,1}),1);
 filename_lane2 = '2500bp.dat';
fid_lane2 = fopen(filename_lane2, 'w');

for n_oligo = 1:length(A_sgi130_2)/3
    oligo_seq = A_sgi130_2{3*n_oligo,1};
    S_sequence = A_sgi130_2{3*n_oligo,1};
    for n2=1:length(A2{1,1})
        B2{n2,1} = strfind(A2{1,1}{n2,1},S_sequence);
          if B2{n2,1} ~= 0

[Score,S_align]=nwalign(S_sequence,A2{1,1}{n2,1},'Alphabet',
'NT', 'Gapopen',2, 'Extendgap', -1);
             fprintf(fid_lane2, 'Oligo name: %s\nOligo
sequence: %s\nBead ID:%s\nBead
```

```
Location:%s %s\n%s\n%s\n%s\n\n\n', A_sgi130_2{3*n_oligo-1,1},
S_sequence,A2{1,1}{n2-5,1}, A2{1,1}{n2-3,1}, A2{1,1}{n2-2,1},
S_align(1,:),S_align(2,:),S_align(3,:));
          end
    end
end

fclose(fid_lane2)
fclose(fid_2)
fclose(fid_sgi130_2)
```

After sequence adjustment of erroneous homopolymeric flanking site, read coverage was analyzed. There are correct read distributions of product produced by different amplification methods.

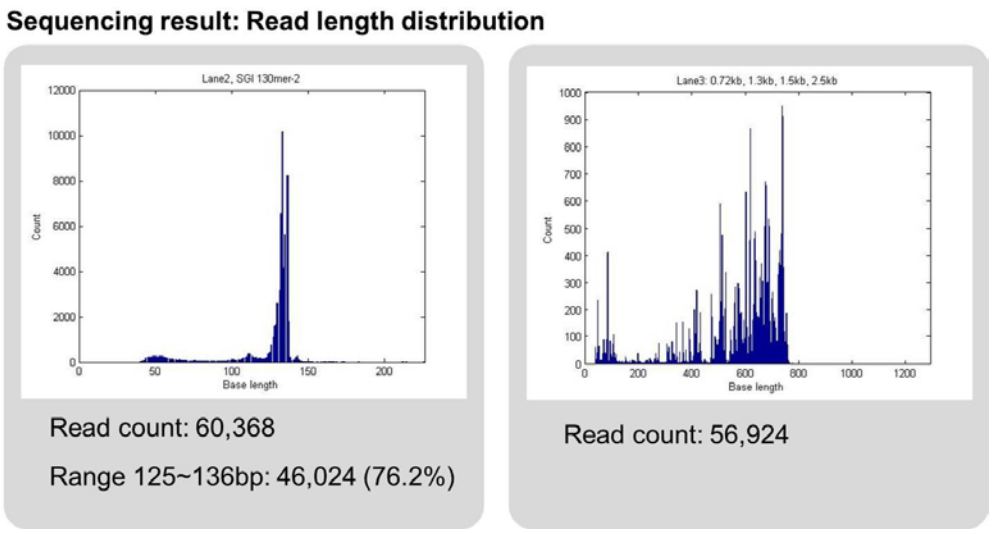


Figure 4.8 Sequencing result of 130bp, and long DNA in 454 sequencing.

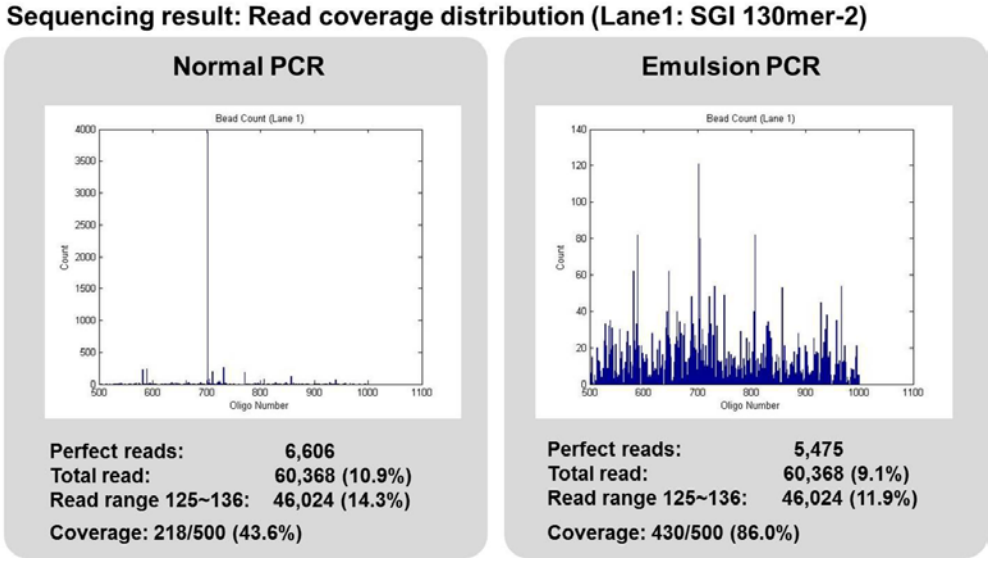


Figure 4.9 Comparison of the sequencing result between normal PCR (Left) and emulsion PCR (Right). Sequencing result from normal PCR shows there is significant amplification bias, but the result from emulsion PCR shows there is much reduced amplification bias.

4.6 shRNA Producing Oligo Design

The RNAi Consortium (TRC) is an academic project that identifies RNA interference function in entire human genome. I download the library file that has shRNA inducing DNA sequences and then sorted for ARS gene panel and ARS interacting gene panel. Double stranded DNA should be inserted in vector for siRNA production. After the sorting of TRC library, universal adapters and ligation sites are tagged. CCGG should be a 5' overhang of forward oligo, but producing sticky end of CCGG is limited in restriction enzyme. Since recognition site of endonuclease is limited, restriction sites are classified into 4 restriction sites. We performed amplification of shRNA inducing oligonucleotides. There is significant amplification

profile difference between normal PCR and emulsion PCR. Band cut and purified products were analyzed with NGS.

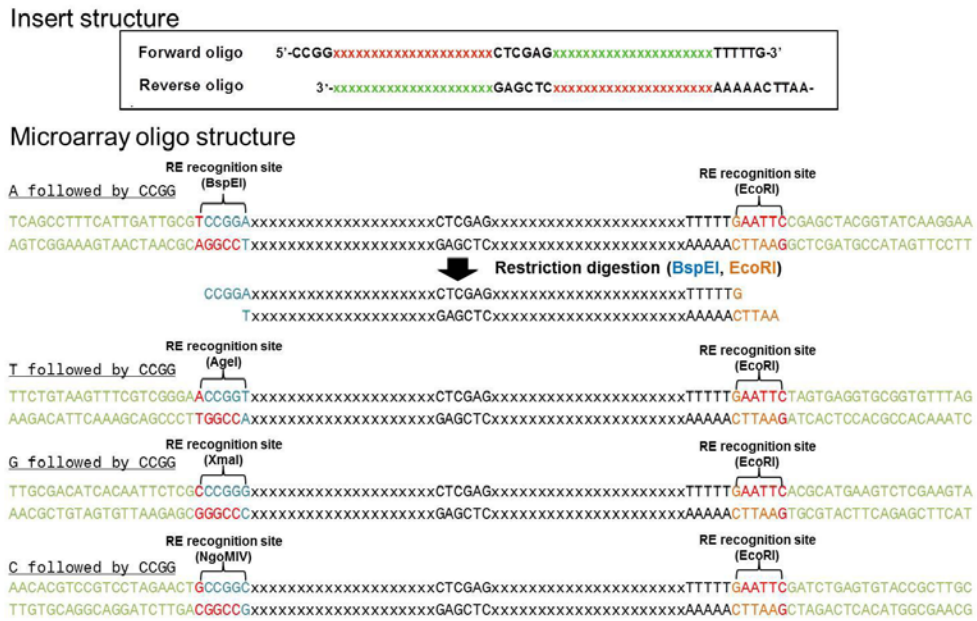


Figure 4.10 shRNA design.

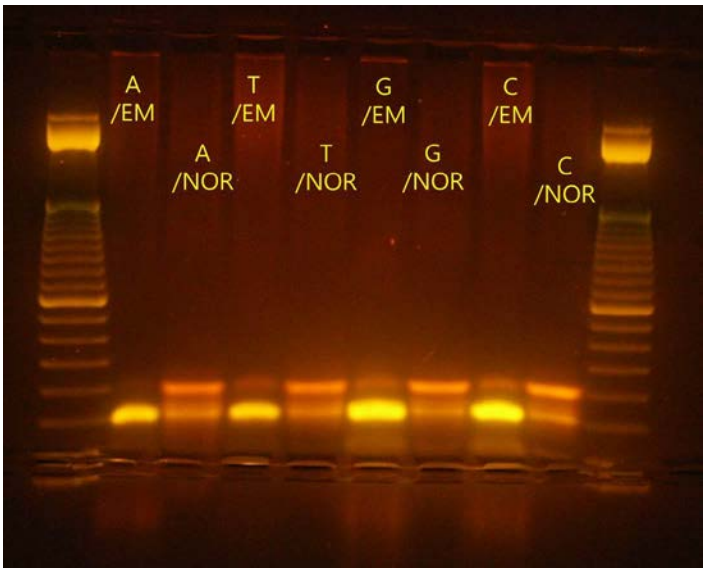


Figure 4.11 shRNA emulsion PCR result.

4.7 Protocol for Emulsion PCR

Materials

- **Oil-surfactant mixture**

Component	Amount	Final concentration
Span 80	2.25 mL	4.5% (vol/vol)
Tween 80	200 uL	0.4% (vol/vol)
Triton X-100	25 uL	0.05% (vol/vol)
Mineral oil	To 50 mL	

- **PCR Mixture**

Component	Amount

Pfu premix 2 (2x)	130 uL
Forward primer (20uM)	13 uL
Reverse primer (20uM)	13 uL
BSA (0.1g/mL)	2 uL
Distilled Water	101 uL
Template ($\leq 10^9$ = 1.66 fmol)	1uL
Total	260 uL

Procedure

Emulation generation

1. Transfer 400uL of oil-surfactant mixture in cryo-tube and magnetic stirring bar
2. (can be skipped) 5 min stirring using rotating magnet, 900 RPM
3. Add 20uL PCR mixture x 10 times (total 200uL) in a drop-wise manner
4. 5 min stirring using rotating magnet, 800 RPM (N.B. : tube centering on magnet rotor)

Thermo-cycling

5. Pipet the emulsion into PCR tubes as 8~10 aliquots of 50uL.
6. Pippete 50uL of the aqueous phase into a well as a non-emulsified control
7. Run thermo-cycler

Step	Temperature	Duration	
Initial activation	95 ℃	3 min	
Denaturation	95 ℃	30 sec	45 cycles
Annealing	55 ℃	30 sec	
Elongation	72 ℃	30 sec	
Final elongation	72 ℃	5 min	
Final hold	4 ℃	∞	

Breaking the emulsion

8. Pool the emulsified PCR reactions in a 1.5mL microcentrifuge tube (8 PCR tubes per 1.5mL microcentrifuge tube)
9. Centrifuge at 13,000rpm for 3 min at 25 °C
10. Dispose of the upper (oil) phase
11. Add 500uL isobutanol in each microcentrifuge (containing eight 50uL emulsified PCR product)
12. Mixing with vortex machine
13. Centrifuge at 13,000rpm for 3 min at 25 °C
14. Dispose of the upper phase
15. (can be skipped) Perform step 11~13 again
16. Perform PCR purification referring to kit manual (ok to use isobutanol instead of isopropanol for the purification additive: add 340ul PCR binding buffer + 300ul isobutanol, and vortexing, then column purification)

Chapter 5

Non-contact Retrieval of Molecular Clones

5.1 Pulse Laser based Molecular Clone Retrieval System

Figure 5.1 shows the schematic and picture of pulse laser retrieval system. Two very accurate, computer controlled motorized stages face each other holding NGS plate and PCR plate respectively. We randomly picked several cornerstone beads from local area. By Sanger sequencing of those cornerstone beads and comparing them with sequence information of NGS, we can link virtual location of sequence information to real world coordinate. Due to its imaging distortion of NGS machine, it is almost impossible to globally map real world location of wanted sequence beads. However, error from imaging distortion can be minimized in relatively small area where real world location of sequence verified microbead is predictable using linear

transformation algorithm. Thermal expansion or radiation pressure of focused pulse laser plays a major role to retrieve microbeads that contains sequence verified oligonucleotides. In contrast to pick-and-place method, owing to non-contact nature of laser light, we can easily avoid cross-contamination and prompt retrieval process can be done within few seconds/bead even with non-optimized prototype.

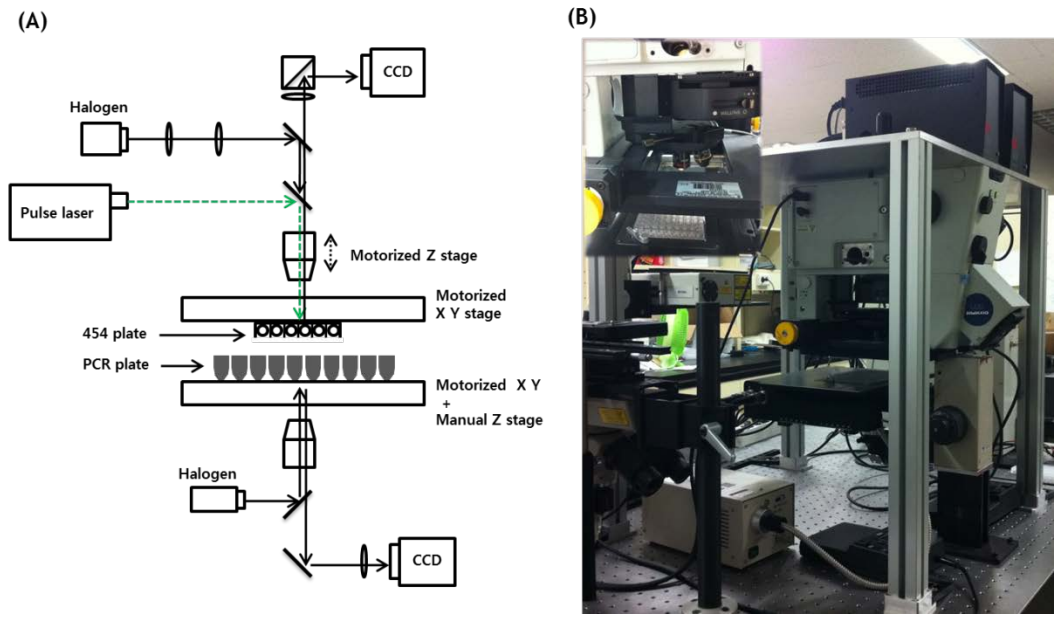


Figure 5.1 Work flow and system construction. (A) Layout of the system. (B) System construction. (1) Target sequences are fragmented in silico and the fragments are synthesized on microarray in parallel manner. (2) Microarray oligonucleotides are amplified by emulsion PCR. (3) Amplified oligonucleotides are sequened by NGS technology and 454 sequencing is used in our case. (4) After sequencing, error-free oligonucleotides are retrieved from NGS substrate plate. To do this, mathematical mapping between sequencing data which give location of each sequenced read and NGS substrate plate. (5) Retrieved oligonucleotides are assembled to error-free long target sequence in test tube

The bright spots of transmission microscopy of NGS chip clearly shows the

physical retrieval of sequence verified oligonucleotide beads (figure 5.2).

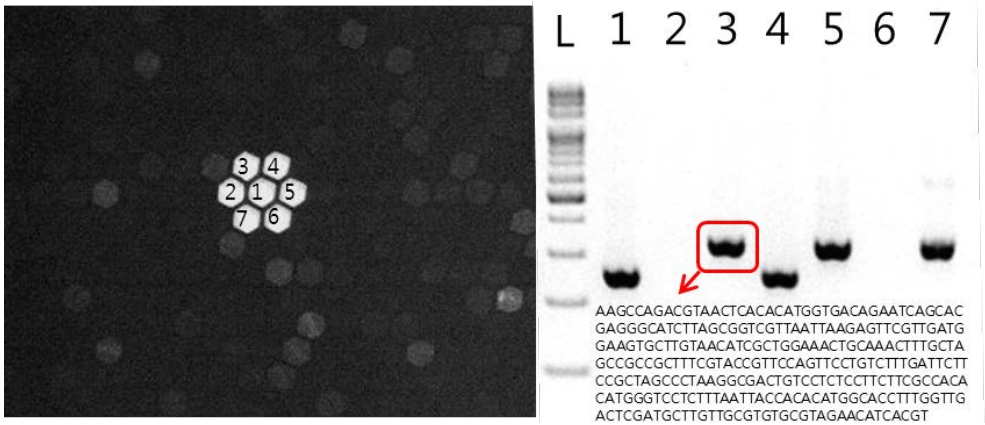


Figure 5.2 Target sequence bead retrieval. Target oligonucleotide is extracted from NGS sequencing plate (left). 1 is targeted and 2-7 which are around the target are extracted. Retrieved oligonucleotides are PCR amplified and visualized by gel electrophoresis (right). Sample 2 and 6 have no DNA template.

As explained before, prediction of real world location of object bead in local area is possible by using cornerstone beads and mathematical transformation function. To see feasibility of our system, we locally map the location of several beads which contains oligonucleotides sequences we want. After retrieval of those beads from the NGS plate followed by PCR amplification and gel purification, then oligonucleotides are Sanger re-sequenced to be compared with NGS data. We successfully retrieved microbeads which contains target sequences in local area.

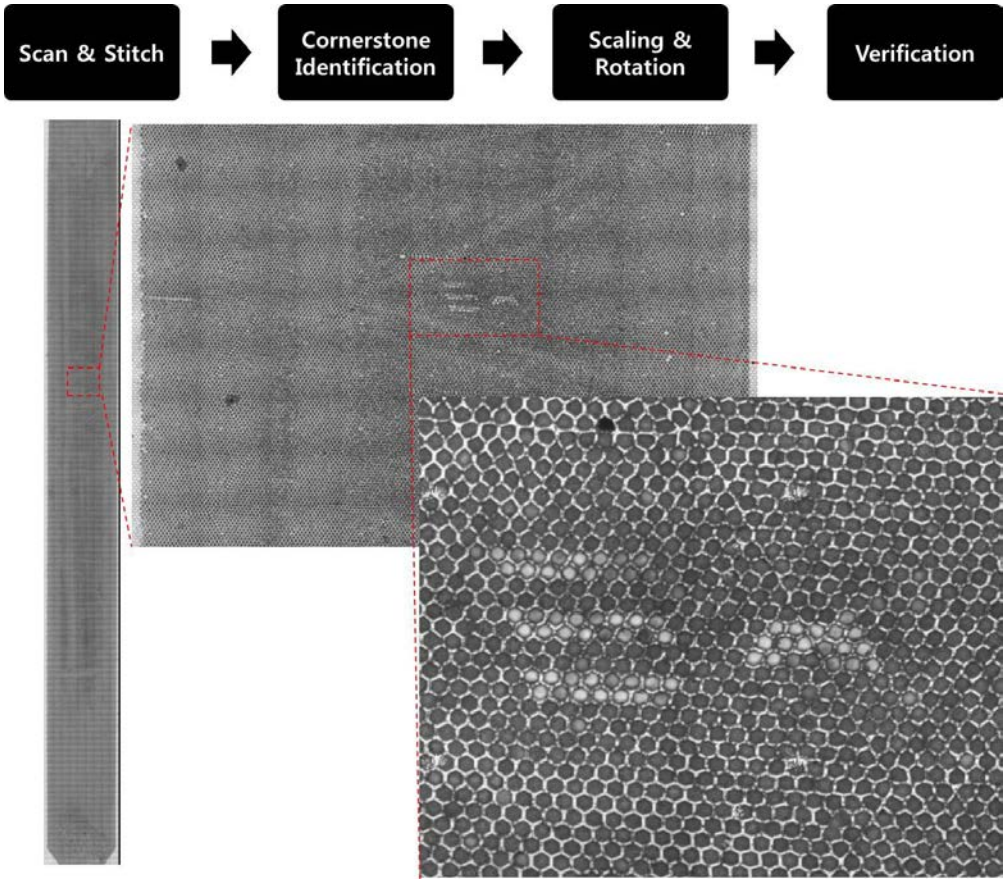


Figure 5.3 Step and scan method to identify PTP wells. Every step image was stored and concatenated for the reconstruction of whole PTP image. Scaling and rotation was performed for the asymmetric location of PTP in the scanning stage.

Every well image was scanned/taken and stitched together. An algorithm for the correction of junction mismatch is incorporated due to the stage error and every scan image is stitched together. In the stitched image, we could identify the position of retrieved beads. These beads are used for identification of cornerstone. Bead PCR and Sanger Sequencing would tell us the sequence information of DNA. Image

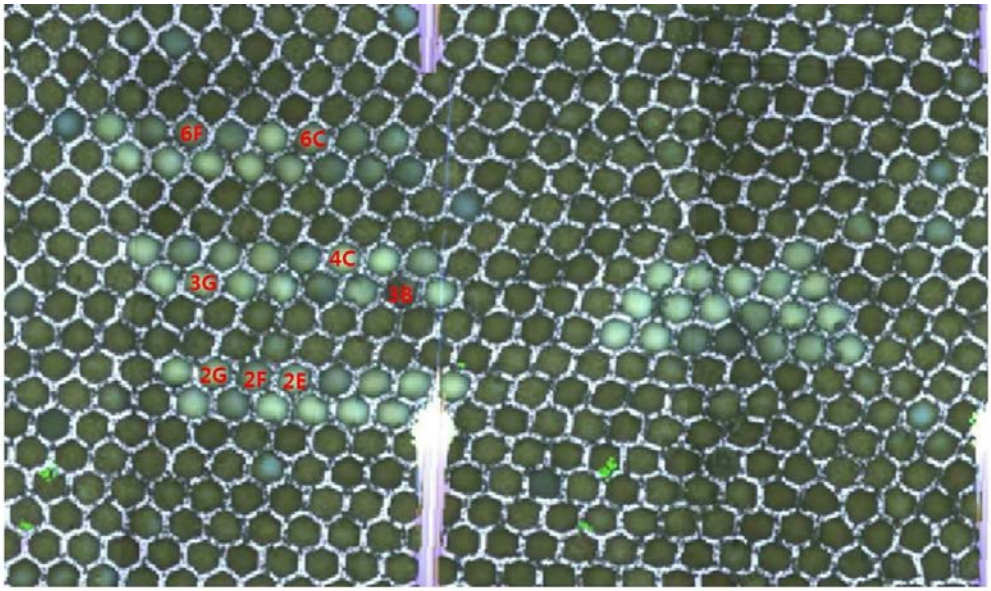
concatenation algorithm was written by MATLAB.

```
clear all

%% Input variables

%Image dimension
x_dim = 1280;
y_dim = 960;

%Number of image
n_image_x = 11;
n_image_y = 10;

% x-axis distortion
x_overlap = 640;
x_y_offset = 6;

y_overlap = 320; %1~2 lane
y_x_offset = 6;  %1~2 lane

y_optimal_range = 30;

y_optimal = 0;

% Write image file name
w_name = 'figure_out\testfig.jpg';
w_ext = 'tif';
```

```
%% Script core
A = zeros(n_image_y*y_dim-(n_image_y-1)*y_overlap+(n_image_x-
1)*x_y_offset,n_image_x*x_dim-(n_image_x-
1)*x_overlap+(n_image_y-1)*y_x_offset,3,'uint8');

n_x = 1;
n_y = 1;

for n_y = 1:n_image_y
   for n_x = 1:n_image_x
      if n_y == 1
          image_name = 'lane';
          image_y_index = num2str(n_y-1);
          image_bar = '-';
          image_x_index = num2str(n_x);
          image_ext = '.jpg';
          image_file =
[image_name,image_y_index,image_bar,image_x_index,image_ext];
          im_call = imread(image_file);
          y_pos = (n_y-1)*(y_dim-y_overlap)+(n_x-
1)*x_y_offset+1:(n_y-1)*(y_dim-y_overlap)+(n_x-
1)*x_y_offset+y_dim;
          x_pos = (n_image_y-n_y)*y_x_offset+(n_x-1)*(x_dim-
x_overlap)+1:(n_image_y-n_y)*y_x_offset+(n_x-1)*(x_dim-
x_overlap)+x_dim;



          A(y_pos , x_pos , :) = im_call(: , : , :);
      else
```

```
            image_name = 'lane';
            image_y_index = num2str(n_y-1);
            image_bar = '-';
            image_x_index = num2str(n_x);
            image_ext = '.jpg';
            image_file =
[image_name,image_y_index,image_bar,image_x_index,image_ext];
            im_call = imread(image_file);

            image_y_index_prev = num2str(n_y-2);
            image_file_prev =
[image_name,image_y_index_prev,image_bar,image_x_index,image_e
xt];
            im_call_prev = imread(image_file_prev);

            image_diff = zeros(y_optimal_range,1);



            for i = -
floor(y_optimal_range/2):floor(y_optimal_range/2)
                im_call_prev_edge = im_call_prev(y_dim-
y_overlap+1+i:y_dim-floor(y_optimal_range/2)+i,1+10:x_dim-
y_x_offset,:);
                im_call_edge = im_call(1:y_overlap-
floor(y_optimal_range/2),y_x_offset+1+10:x_dim,:);
                image_diff(i+floor(y_optimal_range/2)+1,1) =
sum(sum(sum(abs(im_call_edge - im_call_prev_edge))));
            end

            [u,v] = min(image_diff);
```

```
            y_pos = (n_y-1)*(y_dim-y_overlap)+(n_x-
1)*x_y_offset+1:(n_y-1)*(y_dim-y_overlap)+(n_x-
1)*x_y_offset+y_dim;
            x_pos = (n_image_y-n_y)*y_x_offset+(n_x-1)*(x_dim-
x_overlap)+1:(n_image_y-n_y)*y_x_offset+(n_x-1)*(x_dim-
x_overlap)+x_dim;

            y_pos = y_pos + v - floor(y_optimal_range/2);


            A(y_pos , x_pos , :) = im_call(: , : , :);
        end
    end
    n_y
end

imwrite(A,w_name,w_ext)
```



Sample No.	Barcode sequence	Misc.	x position	y position
1B	no match	1B-2, 1B-4	-	-
2E	ACAATTAGTG	ok	1245	1731
2F	GAAAGAAGTC	ok	1242	1730
2G	GAACATCTTT	ok	1240	1731
3B	ATATCGAGTG	ok	1251	1725
3G	GGTATTTTTG	ok	1239	1725
4C	CTATTACGAG	ok	1243	1723
5C	GGCTTAGGTA	single colony	-	-
5G	no match	5G-1, 5G-2	-	-
6C	TACTGTTTAG	single colony	1246	1716
6F	GGCATTCATT	ok	1238	1716

Figure 5.4 Cornerstone bead identification. Beads were retrieved by Laser system and DNA attached on each bead was amplified for the downstream cloning for corner stone sequence identification. Positional information was identified after the sequencing of each clones.

We identified 8 cornerstones. In lane 3 of pico liter plate, we tagged barcode sequence before the template sequence starts. PCR product from bead was TOPO cloned and sent out for sequencing. (Quality of Sanger sequencing from plasmid is superior to that from the PCR product.) PIF file generated in every cycle of 454

sequencing was called. 454 sequencing data come from the image processing of these images. Bright pixels in the combined image of PIF and sequencing data are sequencing pixel data.

```
clear all

%% Read 454 files
filename_1='454reads_2.dat';
fid_1 = fopen(filename_1);
A = textscan(fid_1,'%s');

% Data coordinates
box = zeros(4096,4096);

%% Data extraction core

for n=1:length(A{1,1})/6
    i = strfind(A{1,1}{6*n-3,1},'=');
    j = strfind(A{1,1}{6*n-3,1},'_');
    x = A{1,1}{6*n-3,1}(i+1:j-1);
    y = A{1,1}{6*n-3,1}(j+1:length(A{1,1}{6*n-3,1}));
    box(str2num(y),str2num(x))=1;
    n
end

%% Lane truncation
A_tr = box(:,500:1000);

%% Output
imshow(A_tr)
```

```
imwrite(A_tr,'test_plot2.tif','tif')


%% Image overlay
figure; imshow(im1);
hold on
h = imshow(z);
set(h, 'AlphaData', 0.4)
viscircles(centers1, radii1,'EdgeColor','b','Linewidth',0.1);
```

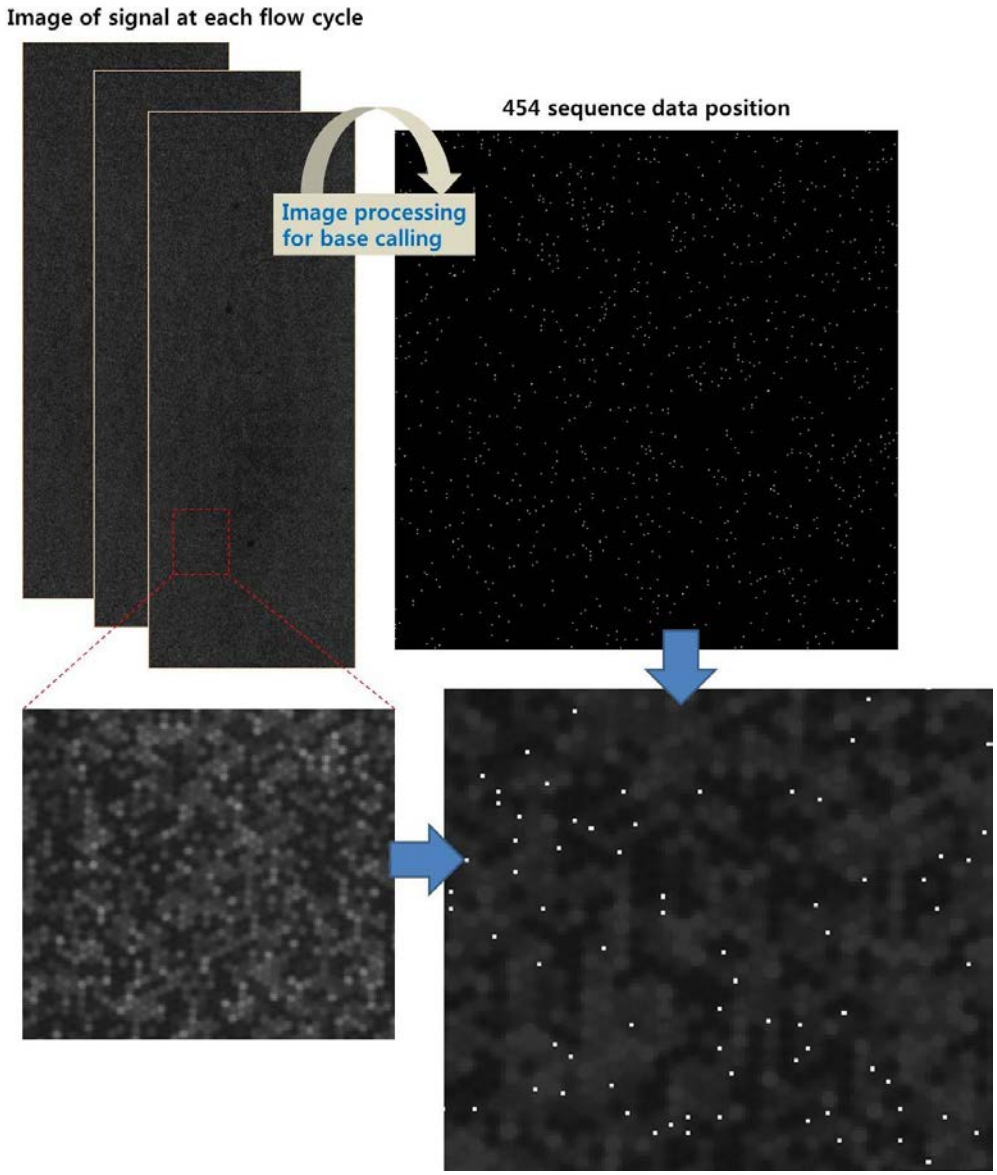


Figure 5.5 Flow signal reconstruction.

Chapter 6

Development of Microfluidic DNA Preconcentrator

6.1 Introduction

Detection of DNA in a microfluidic environment has attracted much attention due to its substantial importance to forensic science, clinical diagnostics, and cancer research. In practice, the short path length of the microfluidic channel and small copy number of the target DNA make sensitive detection challenging. To alleviate these limitations, various techniques have been reported in a form of miniaturized systems. [31-43] The conventional way to enhance the sensitivity of detection is to increase the copy number of DNA by chemical amplification through polymerase chain reaction (PCR). [31-33] However, various components are necessary in PCR chemistry, such as enzymes and primers as well as precise temperature control. It

may also be necessary to incorporate subsequent purification steps before analysis, which adds additional complications to miniaturization. One promising alternative to sensitive detection of DNA would be the capability to concentrate the sample DNA prior to the analysis without any additional chemical involvement.

Sample preconcentration exploits various sample characteristics, including electric charge, size, mobility, and affinity. [34-43] DNA has the unique property of having a uniform charge to size ratio, making charge selective preconcentration efficient. Among the preconcentration techniques using the electric properties of DNA, the electrokinetic trapping technique has received much attention due to its high sensitivity and easy miniaturization. Electrokinetic trapping usually utilizes a nanochannel (or nanoporous membrane) that has an inherently fixed charge, allowing for enrichment of counter ions along the structure while excluding co-ions. [41-43] The local electric field gradient and concentration polarization are induced when an electric field is applied across the charged nanochannel so that sample preconcentration can be achieved. The electrokinetic trapping technique does not suffer from overall device size, since the preconcentration occurs in the immediate proximity of the ion-depletion boundary, and has shown a high efficiency for concentrating charged biomolecules. The conventional fabrication process to incorporate an ion-permselective membrane into a microfluidic device involves complex steps such as multiple masking and subsequent reactive ion etching (RIE).

Furthermore, conventional contact mask lithography may suffer from diffraction mediated blurred pattern when exposing micrometer-sized pattern through the millimeter-thick glass substrate, which reduces spatial resolution. Previously, an optofluidic maskless lithography (OFML) system was developed to provide dynamic in situ polymerization in a microfluidic environment. [44-46] In the OFML system, a computer-controlled spatial light modulator dynamically generates an arbitrary pattern of ultraviolet (UV) light, reducing the complex polymerization process by doing away with the necessity of physical photomasks and mask alignment. In this paper, we present a miniaturized biological sample preconcentrator using charge selective hydrogel, poly-AMPS (2-acrylamido-2-methyl-1-propanesulfonic acid) via OFML. Additionally, a three dimensional optical characterization of the charged hydrogel and preconcentration profile is presented.

6.2 Materials and Methods

Reagents: 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone, N,N′-methylenebisacrylamide, 3-(trimethoxysilyl) propyl methacrylate (TMSMA), glacial acetic acid, potassium phosphate dibasic, and fluorescein disodium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl alcohol, isopropyl alcohol (IPA), potassium chloride (KCl), sodium chloride (NaCl), sodium dodecyl sulfate (SDS),

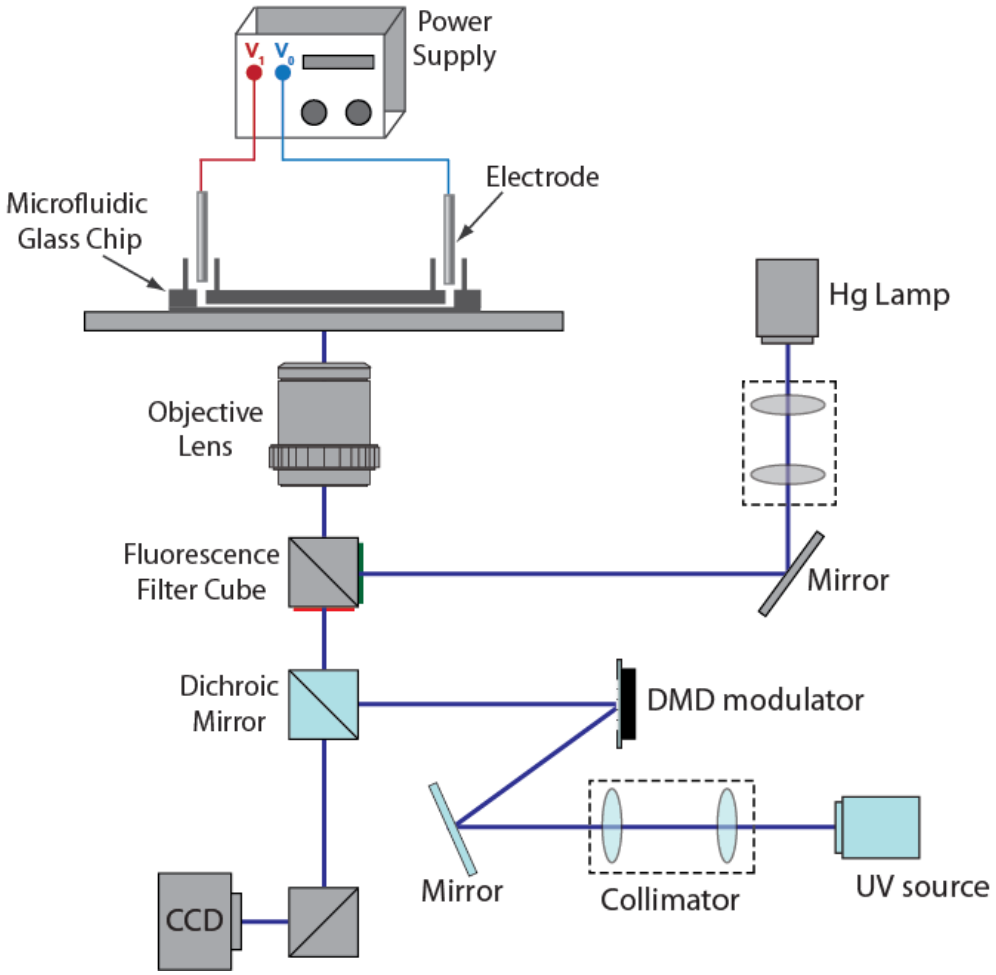


Figure 6.1 Schematic illustration of instrumentation for charge selective preconcentration. A negatively charged hydrogel is produced using the OFML system. A spatial light modulator (DMD) used within the system produces dynamically patterned UV light with arbitrary shape.

hydroquinone (HQ), and sodium hydroxide (NaOH) were purchased from Daejung Chemicals (Korea). All materials were used as received without further modification. De-ionized (DI) water was prepared using Milipore's filtration system (Billerica,

MA, USA). Fluorescent oligomer (Sequence: 5'- Cy3 – GAA GTT GTA GAG TGT -3') was purchased from IDT (Commercial Park Coralville, IA, USA). Buffer solutions were 20 mM phosphate buffer at pH 9.3 unless otherwise stated. We used 100nM Cy3 labeled DNA solution in 0.1x TE buffer mixture (1mM Tris, 0.1mM EDTA, 20mM NaCl, and 0.05% SDS) for the preconcentration analysis.

Microfluidic chip fabrication: Fabrication of the glass microfluidic chips is based on the previous work. [43] The devices are fabricated by standard photolithography and wet etching on glass substrate, followed by thermal bonding to seal the microfluidic channels. The dimensions of the microfluidic channel are 50um (width) and 12um (height).

Optofluidic maskless lithography (OFML) system: A schematic illustration of an optofluidic maskless lithography (OFML) system is shown in Figure 1. The system includes an optical microscope with fluorescence filter cubes (IX71, Olympus), UV source (200W, mercury-xenon lamp, Hamamatsu) and a digital mirror device (DMD, Texas Instrument). UV light from the UV source passes through a beam collimator, prior to redirection by the DMD. The pattern of UV light is controlled by a DMD with self-designed computer program, which synchronizes the pattern of the DMD and the incident UV light. Patterned UV light travels through the side port of the microscope and reflects off a dichroic mirror. The pattern of UV light is focused by passing through the objective lens after which point it exposes on a given substrate

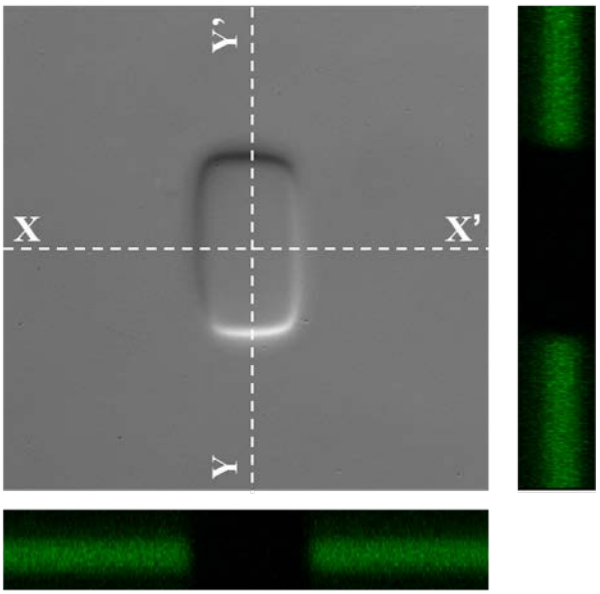


Figure 6.2 3D optical characterization of a negatively charged hydrogel produced by optofluidic maskless lithography.

of interest. The size of a micromirror in the DMD is 13.68 um x 13.68 um, so the calculated ideal resolution of each micromirror after focusing is ~0.7 □m when using 20x objective lens with numerical aperture (NA) of 0.45.

Poly-AMPS fabrication: Prior to the fabrication of poly-AMPS, TMSMA is coated inside the microfluidic channel to covalently link the substrate and the poly-AMPS. 200 uL of TMSMA is diluted with 500uL of DI water and 300 uL glacial acetic acid. The microfluidic channel is filled with the dilute TMSMA mixture for 3 min in a dark environment, then washed with IPA and dried. The monomer solution for poly-AMPS fabrication is prepared with 2 M AMPS in DI water, 2 wt% 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone, 2 wt% N,N′-methylenebisacrylamide, and

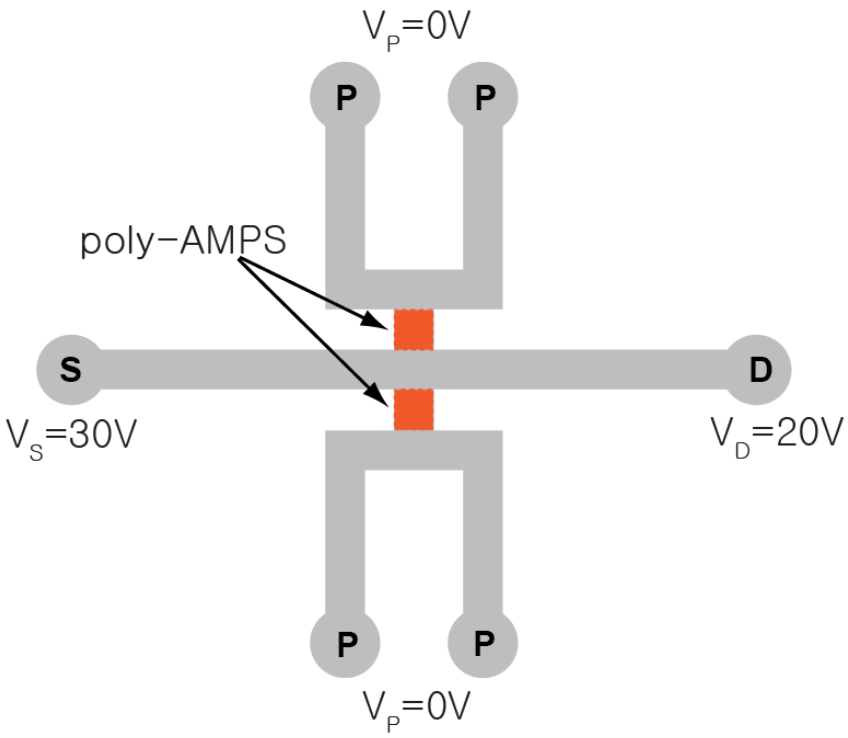


Figure 6.3 Microfluidic channel design for charge selective preconcentration using negatively charged hydrogel, poly-AMPS. The charged sample is injected into the main channel (S-D) while potassium phosphate buffer is added to the polymer channel (P).

200ppm HQ. The monomer solution is flowed into the microfluidic channel, and then a UV pattern is generated by the OFML system and exposed on the microfluidic channel containing the monomer solution. Patterned UV light with energy of 6.8W/cm2 is exposed through the 20X objective lens (Olympus, N.A.=0.45). After polymerization, the microchip is washed with a 1 M KCl solution.

Chip manipulation: 0.1 N NaOH is flowed through each microchannel for 3 min to enhance electro-osmotic flow (EOF). Two power supplies (IT 6720, Itech and

E3633A, Hewlett Packard) are used for voltage application via Pt electrodes.

Optical system: Optical micrographs are acquired by a true-color charge coupled device (CCD) camera (DP71, Olympus) which is directly aligned to an inverted microscope (IX71, Olympus) with a high pressure mercury lamp. 3D fluorescent micrographs are acquired using a confocal microscope (CTR6500, Leica).

6.3 Results and Discussion

The three dimensional (3D) morphology of the poly-AMPS structure fabricated by the OFML system is analyzed using a confocal microscope. The 3D shape of the charged nanoporous structure is important because slight asymmetry or sharp edges along the polymeric wall can affect the electric potential distribution near the charged hydrogel so that the overall preconcentration profile changes. To characterize this, we inject the monomer solution into the microfluidic channel, and expose the patterned UV light of 50 um by 100 um within a wide-open region of the microfluidic channel. The polymerized structure has a height of 12 um; the same height of the microchannel. After polymerization, uncured monomer solution is washed with 1M KCl solution, then the solution of 10 uM fluorescein sodium salt in 20 mM phosphate buffer is added to the channel opening. The negatively charged fluorescein molecule cannot diffuse into the negatively charged poly-AMPS network, which allows us to monitor the 3D morphology via confocal microscope. Transmission microscope image and the corresponding vertical cross section image

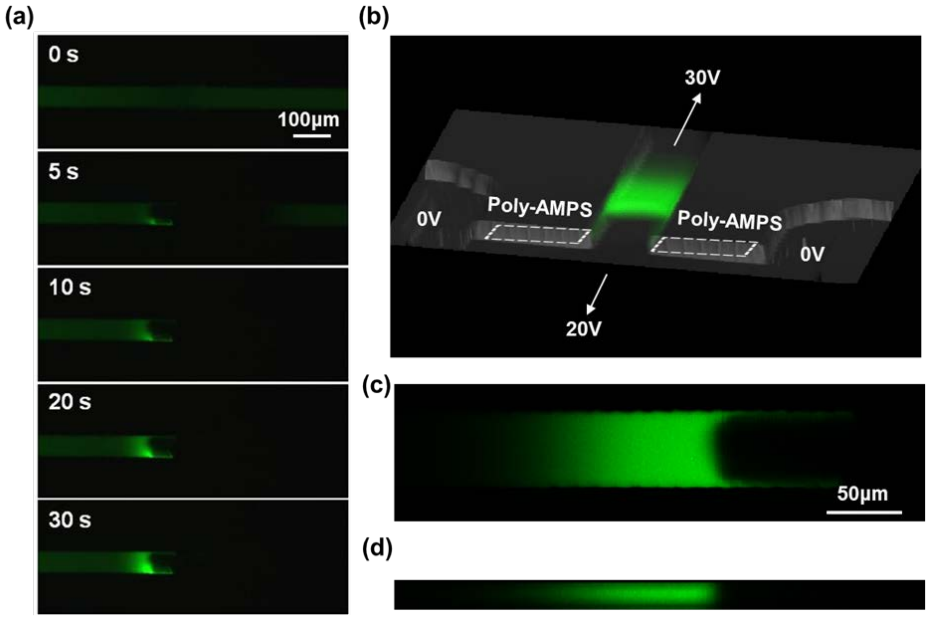


Figure 6.4 3D characterization of sample preconcentration. Negatively charged fluorescein molecule is used for analysis. (a) Time dependent sample preconcentration profile. (b) 3D confocal microscope image after 1 hr of preconcentration. (c) Top view of preconcentration profile (b). (d) Cross sectional side view of (b).

of the polymeric structure are shown in Figure 2. The 3D fluorescent confocal microscope image reveals that the polymer structure produced by OFML system has smooth and vertical wall profile. The OFML system provides sufficient depth of field in a confined channel with 12 um height.

Design of the microfluidic chip for the preconcentration of the charged molecule is shown in Figure 3 (a). Using the OFML system, we produced two poly-AMPS structures in the polymer channel (P) adjacent intersection with the main channel (S-D). In order to measure the concentration of fluorescent molecules, 10 uM

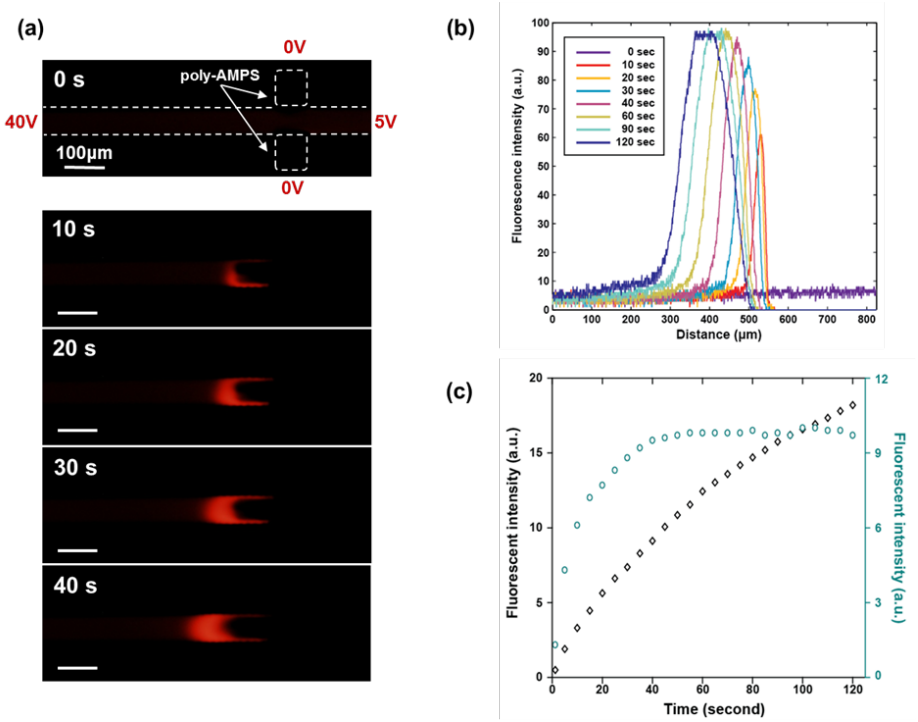


Figure 6.5 Preconcentration profile of DNA. Cy-3 labeled 15 bp ssDNAs are concentrated adjacent to the ion-depletion region. (a) Time dependent sample preconcentration profile. (b) Fluorescence intensity across the main channel at the middle. (c) (Left y-axis) Integrated fluorescence intensities over the entire preconcentrated plugs, (Right y-axis) peak fluorescence intensities of preconcentrated plugs.

fluorescein sample is loaded into the main channel (S-D), while buffer solution is loaded into the polymer channel (P). Fluorescein solution flows through the main channel when voltage is applied to both channel ends. Detailed explanations of the preconcentration mechanism can be found in previous work. Briefly, when P reservoirs are grounded to 0 V, cations are selectively extracted through the negatively charged hydrogel, and anions expelled from the area near the negatively charged hydrogel. As a result, an ion-depletion region develops between the two

hydrogel plugs. Along the ion-depletion region, the electric resistance and corresponding electric field substantially increase. Therefore, electrophoretic force (EP) is locally strengthened at the ion-depletion region. Near the ion-depletion region, the direction of anion EP is the opposite of the EOF of the main channel, thus anions entering the ion-depletion region experience an enhanced EP that drives them back toward the sample reservoir. Consequently, anions are stacked to the left of the ion-depletion boundary where the main channel flow rate and the EP balances each other.

To characterize the local concentration of charged molecules in our device, we applied 30V at the sample reservoir (S), 20V at the drain reservoir (D), and 0V at the polymer reservoirs (P). The concentration dynamics are shown in figure 4 (a). One can see the local concentration of negatively charged fluorescein increases as time passes. 3D optical characterization performed after 1 hour of preconcentration via confocal microscopy (Figure 4 (b)-(d)) clearly illustrates an increase of the fluorescein concentration prior to the ion-depletion region at the channel intersection. Figure 4 (c) is a horizontal cross section of the concentrated region. Concentration of the fluorescein is highest near the ion-depletion region and decreases as the location approaches the sample reservoir (S). Figure 4 (d) is the vertical cross section of the concentrated region. A 3D fluorescence image reveals that the local concentration of the charged molecule is highest at the center of the vertical channel and the profile is

symmetric across entire 50 um width of the microchannel. The preconcentration process is stable even after 1 hour of accumulation.

Lastly, we demonstrate the preconcentration of the DNA. 100 nM Cy-3 labeled single stranded oligonucleotides of 15 base pairs in 0.1x TE buffer mixture is flowed into the main channel (S-D). We applied 40V at the sample reservoir (S), 5V at the drain reservoir (D), and 0V at the polymer reservoirs (P). The dynamics of the DNA concentration are shown in Figure 5 (a). At time zero, before the voltage application, the fluorescence signal is very weak. However, a strong fluorescent signal is detected at the left side of the ion-depletion region after the application of voltage. The spatial distribution of the local concentration along the main channel at the center can be seen in the figure 5 (b). The fluorescence intensity increases linearly for 20 s and then levels off, showing saturation after 40 s (up to ~50-fold). However, the size of accumulated plug increases, keeping the preconcentration rate constant (Figure 5c). The results reveal that DNA can be rapidly and effectively concentrated using the OFML-based charged nanoporous hydrogel.

Chapter 7

Conclusion

This thesis showed a high-throughput technique for the manipulation of molecular clones. With the integration of the recent advancement of highly parallel sequencing and synthesis of DNA, thesis introduces a method to create highly diverse molecular clones, to analyze individual molecular clones, and to retrieve the molecular clones. Method and system developed here may be applicable in a wide range of biotechnology area such as synthetic DNA production, antibody drug discovery, and functional genome study with exceptional cost reduction and increased throughput due to highly parallel nature of the method.

Appendix

120303_snu_130mer 17 tcttagcggtcgttaattaaTACTATAAATTCTACAAGGATTGTACAATACTCATATGGCATACAGGGCAGAAGATTAGATTTATAAACGCCAATTAGAAAATGCAAAAAAGAAGAGTTGGAAGGCTATG

120303_snu_130mer 2 tcttagcggtcgttaattaaAAATAGGAATAACCAACAATAAAGCCTATCCCATGCAAAAAAGGATATTGCCACATGTTAAATAGGAATAACCAACAATAAAGCTTAACCAGAAGAGTTGGAAGGCTATG

120303_snu_130mer 3 tcttagcggtcgttaattaaTTCGAATATCAAAAAGTAAAGGAAATTCTATTTGGTCTGATTCTCGCGACTGACAGGCGAGTCGACAGCTTTCAGGATATACCTGGGTTGAGAAGAGTTGGAAGGCTATG

120303_snu_130mer 4 tcttagcggtcgttaattaaCTCATGATTGTGCAGTCGACGATTAGGAAAATATGTCCACACATCGTGTGTAAGTGAGCTAAACATGATACATGAACTCCATCTCACCCGAGAAGAGTTGGAAGGCTATG

120303_snu_130mer 5 tcttagcggtcgttaattaaTTGACTTGAGCAAGATCAGTCTTATAGCCAACCTGCGCAGAATGGAGGGATCCTTTTTCCAAAAAGAGTTCATTCATTCAATTTTTTTCAAGAAGAGTTGGAAGGCTATG

120303_snu_130mer 6 tcttagcggtcgttaattaaGACAGTCGGCGAGGCGTTGTTCTCTCCAGATTACGAATGTCGGTAACGAAAACGTTGCGCAAGACTTACATTAACTGTAAATTAATGACCAGAAGAGTTGGAAGGCTATG

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120303_snu_130mer 8 tcttagcggtcgttaattaaGATGGTAAGGGTCATTCATTGGAAAGGACATTGTGCGATTCTTTTAGCTTACAAACTGTTTTCCTACAGGACATTTAGCCCTTGTACTGTAGAAGAGTTGGAAGGCTATG

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120303_snu_130mer 939 CGATGAACTATCGCTCTTCTATCACTCTGCTTCCCATCCTGCTTTCATTTGCCCGTAGAAAGCTGAAGCAACCCGCGTTAGGGAAAGAGACAGCCACCACCGTAGACCAAttaattaaccacacatggca

120303_snu_130mer 940 CGATGAACTATCGCTCTTCTTGGCTGCTCCAATATTCCTTCACTGTCAATTCAACCCAAGCCAGCATGGCTCCGTCGCTCCCCGAACCTGTGCCAGATTCGCCCTCTGACttaattaaccacacatggca

120303_snu_130mer 941 CGATGAACTATCGCTCTTCTACGTTTACGCTGTATCTTATTCGTCACGGCGAAGCTAGTCACAACGTGGAAGAAAAGCTAGCACAAAAGCAGGCTCTTGAAGAATGCATCTttaattaaccacacatggca

120303_snu_130mer 942 CGATGAACTATCGCTCTTCTGACGACCCCGTCACGAAGGAAAAAATGGAACAAGCCAGACAAGCCATTCTCGAAAATCCCGACTTTTTCGATGCTCGTCTTTCCGATCTCttaattaaccacacatggca

120303_snu_130mer 943 CGATGAACTATCGCTCTTCTGCAGCCCGAGCCCTAATCGAAACGTGGGGCGATTTGCCTGTTCCGCAGGAGGTGTTTGTCAGCCCTTTGCAGCGGACGCTACAAACCGCTttaattaaccacacatggca

120303_snu_130mer 944 CGATGAACTATCGCTCTTCTCATCCCAATATTCACGTCCGAGAAGAGTTACGGGAGCGACTAACCGGACGTCCCGCCGACAATCGATTTTCCAGCACAGAACTCAGTCGTttaattaaccacacatggca

120303_snu_130mer 945 CGATGAACTATCGCTCTTCTTTTTCCTTTAAGCATTTGCGACTCAACTCCATTGTGAATGATCTGGCTGGAATTTTTGGAGGAACAAGTTCCACCGATTTGCAAGAATTGttaattaaccacacatggca

120303_snu_130mer 946 CGATGAACTATCGCTCTTCTGACGAAGATTTGTCGGGCACCGCTCGGAGCAGCGTGGTTAGCTTCGAGAGCAACTCTAGTTTACATCATAATGTCACGAATACACGCAGCttaattaaccacacatggca

120303_snu_130mer 947 CGATGAACTATCGCTCTTCTTCGTGCAACGCCGACGAAGTCGTGGAAGACGAGGCCAAACTCCGCGAACGGACTTTCAAAGTGTTTAAGCTTCTCCTCGAGTCGGAACACttaattaaccacacatggca

120303_snu_130mer 948 CGATGAACTATCGCTCTTCTACTCATAAGGGTTATTTGCGAACCTTGGAACGGGGGCATTTGCATCAACCAAATGCGCGGCAGTTTCAGAATTGCGAAGTTCGTGTTTACttaattaaccacacatggca

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120303_snu_130mer 950 CGATGAACTATCGCTCTTCTTTGTGAAAGAGCAATAACGTGGGCGGCAATCCTTCGTAAGCCTGTTATTGGGAAAGTTCTTTCGCCCCTGGCGTCAATAGACTACAGACAttaattaaccacacatggca

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120303_snu_130mer 962 CGATGAACTATCGCTCTTCTATTTCGGTTTTTTAGTAGAGTTCGACCCGATTTACATCACATCAGCATAGTTCTAATGTAACTGTAAGTGTTTTTAGGATTACAAATACGttaattaaccacacatggca

120303_snu_130mer 963 CGATGAACTATCGCTCTTCTTTCCCTCGGACTCAATAAGGAGCAACACTCGTGGATGCTGGATTTAACAAGGCATTCATCGAATGGCTCCCGTTACTTTGCTATTACTTTTttaattaaccacacatggca

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120303_snu_130mer 968 CGATGAACTATCGCTCTTCTGTGACAGTAAATTTTTCCATTCCGCTCTCCTTTTATCACCAGCAAGAAAATCCACGGGAATGGACTATTCAGGTAGCGACCGAGGCATTGttaattaaccacacatggca

120303_snu_130mer 969 CGATGAACTATCGCTCTTCTTCACGGGGTTTAGTCGGGTTGCCCCTTCACCAGATTCAGCAGCAGGGCTCGAGCATTTCTCCTTGCGAAAATTCGCAATCCAGAGATGGTttaattaaccacacatggca

120303_snu_130mer 970 CGATGAACTATCGCTCTTCTTGTTCATTAGGTCGTGCCATTCAAAAAATGAATTTAATGCTGTCAAATCTCGAAAGCGATTGGAGGGATATCTCACACTCAGGACTCAACttaattaaccacacatggca

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120303_snu_130mer 972 CGATGAACTATCGCTCTTCTGGGCCGTCTCGGTCTACATACACGTTATCTACGCGTTTAATACGTAAAGTTGTAAATGGGGAGTGCGAGGCTGACGGGTTGTTGTCCTCCTttaattaaccacacatggca

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120303_snu_130mer 974 CGATGAACTATCGCTCTTCTCGGATTTTGGACAAACTCTTGCTGCGGCGAGACTATAATCCCGCGCACAAAGCGAAGGGAATTCGACGATCTGGATGTGTAAGTCTAACCttaattaaccacacatggca

120303_snu_130mer 975 CGATGAACTATCGCTCTTCTGCTGCACCGTCAGCTGATGTGAATAGCGCTATGGTTTTTTCACTTCGAGCAACTGTTAAAGGACTCCGCTTACCTGCCGGCTGATAAAAGttaattaaccacacatggca

120303_snu_130mer 976 CGATGAACTATCGCTCTTCTTCCGGCCTCGATGAGGCCGCAAAATCACTATTCCGGCCAAAATCAAGATTTGCTTACAGTTAGTACCAAAGGTGGGGCCGCATTACTTCCttaattaaccacacatggca

120303_snu_130mer 977 CGATGAACTATCGCTCTTCTAAGAAATATGGTTTAAAGCCTGTCGATGGAAGATGTTCTCCAAACGACGCTTCGTCCGCAGGAAGCATCTTGAGAGATGATCCATGGATTttaattaaccacacatggca

120303_snu_130mer 978 CGATGAACTATCGCTCTTCTCTTGAGAATGTAACTTTCAAGTTCATTAATCGTGTGTGATCCAATTACTTAGGAAACAACACTGTCCCTATCTTAGCTACTCAACGAAATttaattaaccacacatggca

120303_snu_130mer 979 CGATGAACTATCGCTCTTCTAGTGATTTTGCGGCCGCATCGAGGCGAGGATCAGTGATTTTCCTCTAAAAATTGAATTTAGCAAAAAGTCCCTTCTTGCTAGGTTTTAGGttaattaaccacacatggca

120303_snu_130mer 980 CGATGAACTATCGCTCTTCTAGGGGTAAGAAATACCTCTAATGGTCCAGTGTAGCTTACTGTTACTGTATATAATTTTCAATCTCAGCTCTTGTCCGTTGGGTATCGAGAttaattaaccacacatggca

120303_snu_130mer 981 CGATGAACTATCGCTCTTCTGATTTTGCAGTTAGCCATCGAAGCTGACTGTGAGTTTTTGTATGTCTGTTAATTCGTTCCCGCCAATTATGGTTAGAAAACCTACCATTTttaattaaccacacatggca

120303_snu_130mer 982 CGATGAACTATCGCTCTTCTTTTCTTTTGAATACTGCCCATAGGATGGAACAGGCAGTTCACCTTTGGGTCTTTTTGGAAAAGCAATATAACAAATTTCAGTGAAGATCAttaattaaccacacatggca

120303_snu_130mer 983 CGATGAACTATCGCTCTTCTTTCTTTTTGTATAATATTAGTTCCTATCGCAACGGATCAAAATTTGTTGTCAAACTGACAGCACAAGTTAGTTAGTTTTGGGTCTTGGGTttaattaaccacacatggca

120303_snu_130mer 984 CGATGAACTATCGCTCTTCTCCATGATTGACTGTACAAAGAAAACCACACAGGGGATCCAGCAAAAAGCGTTGCAAATCTGTGAATACATCAACGGTTTCCCCTTACTACACttaattaaccacacatggca

120303_snu_130mer 985 CGATGAACTATCGCTCTTCTCGTTTGGTAGATTATTTCAAATCCCCAGCAACAACGAAACAAGGAAAGAATTAAGGACAACATCTTCCACAGCCCATGCTCAATCGTCGAttaattaaccacacatggca

120303_snu_130mer 986 CGATGAACTATCGCTCTTCTTTTATGAGCATGAGAGCTCTTCTTTTATCGGCCGCGATCAGCCTGTTTCCATGTGCATGGGCCTTTTCAGATATAGGATACGAGGCGACGttaattaaccacacatggca

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120303_snu_130mer 988 CGATGAACTATCGCTCTTCTCGCATGGCCGGAGAAAACTTCACGGAAAAGCGAAATGTTTATGATAATGGACGCAACTCGGCGTCTATGACATTAAGAGGATTGAGTATTttaattaaccacacatggca

120303_snu_130mer 989 CGATGAACTATCGCTCTTCTGGCTCCTATCCACTGTATAATATATACCGTTACAGCTTCGAGAAGCTTGGGGAGACAATTGAAGGGGATGATCCCGGATTTTTCGATGGAttaattaaccacacatggca

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120303_snu_130mer 993 CGATGAACTATCGCTCTTCTTTTCTGAAGGTCGATATTGCAAGTGACCGGTGTGCGCAAGGCGAAACAGGTTACGTGGAGATGCGAAAGAAGGTCAAAGTTCTCATCAGCttaattaaccacacatggca

120303_snu_130mer 994 CGATGAACTATCGCTCTTCTGTTCAAAGTCTCATTCATCACATCGAGCAGGTTCAAAATAGTGGCATATCTGATTTTTGCGAACTCTATGCTCTTGCTCTGTATTCACAAttaattaaccacacatggca

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120303_snu_130mer 1000 CGATGAACTATCGCTCTTCTGAACTAAGTTCAGCTATTGATGCTTGTGCAAGTAATTCTACTGCATCTACTGCAGCACAATTATGGGATGGAGGCGTAGCGTTCTTGATAttaattaaccacacatggca

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국문 초록

분자 클론이란 하나의 DNA 분자로부터 복제된 다수의 DNA 분자 군집을 의미하며 하나의 분자 클론 내에 있는 DNA 분자들은 모두 같은 염기서열을 갖는다. 현대 생명공학에서 분자 클론을 생성시키고, 이를 분석 및 추출하는 기술은 가장 기본적이며 핵심적인 기술이다. 본 연구에서는 복사압 기반의 비접촉식 분자클론 기술을 개발하고 초 병렬적 염기서열 분석 방법과 이를 융합시켜 다량의 분자 클론을 빠르게 생성, 분석 및 추출하는 기술을 개시한다.

구체적으로, 초 병렬적 염기서열 분석 방법을 이용해 분자 클론을 생성시키고 분석하는 방법에 대해 소개한다. 초 병렬적 파이로시퀀싱의 샘플 준비 과정에서 사용되는 에멀젼 PCR 을 이용하여 다량의 분자 클론을 생성시킨다. 생성된 다량의 분자 클론의 염기서열은 파이로시퀀싱 과정을 통하여 분석되며 2 차원 기판에 위치한 각각의 분자 클론의 위치와 염기서열이 매핑된다. 복사압 기반의 비 접촉식 에너지 전달 장치를 개발하여 미세한 분자 클론 각각을 빠르고 정교하게 기판으로부터 추출해 낸다. 펄스 레이저, 광학계, 머신 비전, 직각좌표 로봇 등이 융합된 비접촉식 분자 클론 추출 장치가 소개된다. 또한, 초 병렬적 염기서열 분석의 데이터로부터 염기서열의 추출, 2 차원 기판 내 분자 클론의 위치정보를 연결시키는 SW 를 개발한다. 염기서열 분석 기판의

움직임, 펄스 레이저의 조사 및 추출, 마이크로 웰 플레이트의 움직임을 각각 컨트롤 할 수 있는 소프트웨어가 소개된다.

이와 더불어 박테리아 세포의 분열으로부터 생성된 분자 클론의 추출 기술이 소개된다. 유전적으로 다양한 DNA 를 포함하고 있는 박테리아 세포 용액을 고체 지지체에서 키운 후, 현미경 비전 시스템을 이용해 각각의 분자 클론을 확인한다. 생성된 박테리아 분자 클론의 위치를 확인하고 이를 펄스 레이저 시스템을 이용하여 빠르게 추출해 낸다. 추출된 박테리아 클론의 DNA 각각은 바코드 DNA 를 도입하여 한번의 초 병렬적 염기서열 분석 기술을 이용하여 서로 다른 분자 클론의 DNA 정보를 한번에 분석한다.

본 연구에서 소개되는 방법과 시스템을 이용하면 합성 유전자의 생산, 항체 신약 개발, 유전체 기능 연구 등 광범위한 생명공학의 연구에 소요되는 비용을 낮추고 생산성을 향상시킬 수 있을 것으로 기대한다.

주요어 : 분자 클로닝, 유전자 합성, 합성 생물학, 시스템 생물학

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